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(54) Title: INHIBITING APOPTOSIS WITH ADENOVIRUS RID PROTEIN (57) Abstract <p>A method for inhibiting apoptosis of a cell expressing a death receptor of the TNFR family is disclosed. The method involves treating the cell with a Receptor Internalization and Degradation (RID) protein complex containing RIDα (10.4K) and RIDβ (14.5K) proteins encoded by the E3 region of adenovirus. The cell can be treated by administering to the cell a polynucleotide expressing the RID complex or by administering to the cell a composition containing the RID complex. Compositions containing a RID complex are also disclosed. The compositions and method are useful in the treatment of cancer, degenerative and immune disorders, as well as in promoting survival of tissue transplants. An adenovirus vector for delivering the RID complex to cells is also disclosed.</p>		

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Inhibiting Apoptosis with Adenovirus RID Protein

Reference to Government Grant

This invention was made with government support under Grant Number RO1 CA58538. The government has certain rights in this invention.

5 Related Application

This application claims priority to U.S. Provisional Application serial number 60/088,993, filed July 9, 1997, which is incorporated herein in its entirety by reference.

Background of the Invention

10 (1) Field of the Invention

This invention relates generally to the regulation of apoptosis and, more particularly, to a method for inhibiting apoptosis using the Adenovirus RID protein and to applications of this method, including promoting survival of tissue transplants, treating autoimmune disease, and promoting tumor destruction in cancer patients.

15 (2) Description of the Related Art

Apoptosis, or programmed cell death, plays a fundamental role in regulation of the immune system. For review, see White, E. *Genes & Development* 10:1-15, 1996; van Parijs, L. and Abbas, A.K., *Curr. Opin. Immunol.* 8:355-361, 1996; Nagata, S., *Cell* 88:355-365, 1997. In recent years researchers have shown that some members of the tumor necrosis factor

20 (TNF) family of cytokines can induce apoptosis by binding to their specific receptors on

target cells. Nagata, *supra*; Baker, S.J. and Reddy, E.P., *Oncogene* 12:1-9, 1996. The receptors for the TNF family of cytokines belong to a family of proteins referred to as the TNFR family, which is characterized by an extracellular domain of highly conserved cysteine residues contained in cysteine-rich pseudorepeats (Chaudhary et al., *Immunity* 7:821-830, 5 1997). In addition, several members of the TNFR family possess a conserved cytoplasmic domain of approximately 80 amino acids called the death domain, which functions to initiate an intracellular apoptotic signaling cascade upon binding of the appropriate cytokine. (See Chaudhary et al., *supra*; Walczak et al., *EMBO J.* 16:5386-5397, 1997.) TNFR proteins containing death domains comprise a death receptor subfamily which includes: TNFR1 10 (Tartaglia et al., *Cell* 74:845-853, 1993); Fas (also called CD95 and Apo-1) (Itoh and Nagata, *J.Biol. Chem.* 268:10932-10937, 1993); death receptor 3 (DR3, also called TRAMP, Apo-3, Wsl-1, and LARD) (Chinnaiyan et al., *Science* 274:990-992, 1996; Kiston et al., *Nature* 384:372-375, 1996); TRAIL-R1 (also known as DR4) (Pan et al., *Science* 276:111-113, 1997); and TRAIL-R2 (also called DR5) (Pan et al., *Science* 277:815-818, 1997). The death 15 domains of these proteins are shown in Figure 1.

Fas, the most studied death receptor, is expressed on the surface of most cell types, including epithelial cells, fibroblasts, T and B cells, liver hepatocytes and some tumor cells (Nagata, *Nature Medicine* 2:1306-1307, 1996; French et al., *Nature Medicine* 3:387-388, 1997). However, FasL is primarily expressed by activated leukocytes of the immune system, 20 including cytotoxic T lymphocytes (CTL's) and natural killer (NK) cells (Nagata, *Cell, supra*). It is believed that the Fas ligand (FasL) plays a role in the immune response of these cells to induce apoptosis in target cells expressing Fas. Such target cells include virus-infected cells and tumor cells. On the other hand, leukocytes also express Fas, which can result in down regulation of the immune response due to activated leukocytes killing each 25 other (Nagata, *Cell, supra*).

Recently, it was discovered that FasL is also expressed in immune-privileged sites such as the eye chamber, parts of the nervous system, and testis and it is believed that any activated leukocytes entering such sites are immediately killed through the FasL-Fas apoptotic pathway, thereby preventing a potentially crippling immune response (Nagata, *Cell, supra*). This finding could potentially be applied to preventing transplant rejection and, 30 indeed, one group has reported that islet allografts were protected from immune rejection by cotransplantation with syngeneic myoblasts expressing functional FasL (Lau et al., *Science* 273:109-112, 1996).

The discovery of FasL expression in immune-privileged sites led a number of groups 35 to examine whether the means by which tumor cells avoid destruction is through expression of FasL. A number of tumor cell types were subsequently reported to constitutively express

FasL, including lymphoma and leukemia cells (Tanake, et al., *Nature Med.* 2:317-322, 1996) various nonlymphoid carcinoma cells, including colon cancer (O'Connell, et al., *J. Exp. Med.* 184:1075-1082, 1996), hepatocellular carcinoma (Strand et al., *Nature Med.* 2:1361-1366, 1996) and melanoma (Hahne et al., *Science* 274:1363-1366, 1996). As a result of expressing FasL, many tumor cells have the ability to kill attacking CTL and NK cells thereby reducing the immune response against the tumor. In addition, it has been reported that some types of tumors become resistant to Fas-mediated apoptosis, either by downregulation of Fas expression or by other unknown mechanisms, and thereby avoid being killed by the infiltrating leukocytes (Nagata, *Nat. Med.*, *supra.*; Strand et al., *supra.*; Hahne et al., *supra.*). Because alterations in Fas-FasL regulation, including upregulation of FasL expression and downregulation of Fas expression, may be involved in tumor cells avoiding destruction by the immune system, it would be desirable to devise an approach that would reduce the effect of such changes in Fas-FasL regulation. In one such approach it was recently reported that the anti-cancer drug doxorubicin enhances expression of both Fas and FasL in tumor cells (Friesen et al., *Nature Med.* 2:574-577, 1996).

Recent reports have associated other disease states with dysfunction of the Fas system, including hypereosinophilic syndromes in humans (Lenardo et al., *J. Exp. Med.* 183:721-724, 1996), hepatitis (Kondo et al., *Nat. Med.* 3:409-413, 1997) and the autoimmune disease Hashimoto's thyroiditis (HT) (Giordano et al., *Science* 175:960-963, 1997). Consequently, it has been suggested that inappropriate upregulation of Fas may be a causal factor in other autoimmune diseases involving tissues which constitutively express FasL (French et al., *supra.*).

Human adenoviruses (used interchangeably herein with Ad), which cause disease in the respiratory tract, conjunctiva, intestine, urinary tract and liver, have evolved elaborate mechanisms to overcome host antiviral defenses, including at least four of the seven known proteins encoded by the early region 3 (E3) transcription unit which have been reported to inhibit the host immune response to Ad-infected cells (Fejer et al., *J. Virol.* 68:5871-5881, 1994; Sparer et al., *J. Virol.* 70:2431-2439, 1996). One of these proteins is a 19kDa glycoprotein (gp19K), which inhibits CTL-mediated lysis of Ad-infected cells *in vitro* (Efrat et al., *Proc. Natl. Acad. Sci.* 92:6947-6951, 1995). Three other E3 proteins, the 14.7K protein and 10.4K protein in combination with the 14.5K protein (referenced hereinafter as the 10.4K/14.5K complex), protect adenovirus-infected cells against cytolysis and the inflammatory response induced by tumor necrosis factor- α (TNF- α) both *in vitro* and *in vivo* (Sparer et al., *supra.*; Krajcsi et al., *J. Virol.* 70:4904-4913, 1996; Dimitrov et al., *J. Virol.* 71:2830-2837, 1997). Although the exact stoichiometry of 10.4K and 14.5K proteins in this complex is not known, it is believed to consist of one 14.5K polypeptide in physical

association with a dimer formed by full-length and short forms of the 10.4K polypeptide joined in disulfide linkage. Stewart et al, *supra*.

Efrat et al. have reported that the expression of the one of the Ad E3 genes, i.e. the gene encoding the 19kDa glycoprotein (gp19K), can prolong survival of pancreatic islet allografts. The islets were obtained from transgenic animals prepared to contain the entire E3 genomic DNA from human Ad, however, the gp19K mRNA was prominently expressed with little or no expression of the 10.4K protein which makes up a portion of the 10.4/14.5 complex. The islet allografts survived reportedly due to the expression of the gp19K protein and there was no suggestion in this reference that the 10.4K or 14.5K proteins either separately or in the 10.4K/14.5K complex played any role in the survival of the allografts.

Nevertheless, the 10.4/14.5 complex can protect Ad-infected cells from the inflammatory response in the context of Ad infection (Sparer et al., *supra*) and, although it has not been heretofore recognized, it is possible that the 10.4K/14.5K complex could also provide a novel basis for modulating the immune system in certain disease processes.

Summary of the Invention

In accordance with the present invention, the inventor herein has succeeded in discovering that the Ad 10.4K/14.5K complex inhibits apoptosis mediated by death receptors, in particular Fas or TNFR-1, by removing the death receptor from the cell surface. The present invention, thus, provides a method for inhibiting apoptosis of a cell comprising treating the cell with an effective amount of a 10.4K/14.5K complex referenced herein as RID (Receptor Internalization and Death) or as RID complex. The RID complex reduces the number of molecules of one or more death receptors on the surface of the cell. This down-regulation of the death receptor results from internalization of the receptor to endosomes and degradation of the internalized death receptor by lysozymes. The RID complex is obtained from or derived from the RID α and RID β proteins encoded by the Ad E3 region DNA. Other E3 region-encoded proteins, including the gp19K and 14.7K proteins, are not required to remove the death receptor from the cell surface or to induce apoptosis. Due to the similar structure of TNFR death receptors, and in the common pathway by which they mediate apoptosis, it is believed that RID can inhibit apoptosis mediated by all death receptor members of the TNFR family by promoting their removal from the cell surface.

In one embodiment of the present invention, the cell is treated with RID by administering to the cell a polynucleotide encoding the RID complex, through which the RID complex is expressed in the cell. Alternatively, the treating step comprises administering the RID complex to the cell, preferably in a carrier that facilitates delivery of the complex into the cell. The method can be used to inhibit apoptosis of cells expressing one or more death

receptors of the TNFR family, including but not limited to Fas, TNFR-1, DR3, TRAIL-R1 and TRAIL-R2. Where the cell comprises a tissue, the method is useful for promoting survival of a tissue transplant in a patient or in promoting survival of a tissue under attack in a patient suffering from a degenerative disease, an immunodeficiency disease, an autoimmune disorder or other diseases associated with dysregulation of apoptosis mediated by the TNFR death receptors. The method is also useful in inhibiting apoptosis of leukocytes mediated by tumor cells in cancer patients, thereby promoting leukocyte destruction of the patient's tumor cells.

Accordingly, in another embodiment, the present invention provides a method for
10 decreasing apoptosis of target cells in a patient comprising treating the patient with an
effective amount of a RID complex. The target cells express a death receptor which is
downregulated when RID enters the cells.

15 In yet another embodiment, the invention provides a method for inhibiting leukocyte apoptosis in a patient comprising withdrawing leukocytes from the patient, treating the leukocytes with an effective amount of a RID complex, and administering the treated leukocytes to the patient.

In another embodiment, the present invention provides a composition comprising a RID complex in a carrier suitable for facilitating entry of the RID complex into a cell. As illustrated in Figure 3, a RID complex comprises at least three polypeptides: a full-length Ad E3 10.4K protein having two transmembrane domains (RID α -L), a short form of the 10.4K protein with only one transmembrane domain (RID α -S), and a 14.5K protein (RID β). RID compositions intended for treating humans preferably contain a pharmaceutically acceptable carrier. In one embodiment, the carrier component of the composition comprises a liposome.

25 The present invention also provides an Ad vector for expressing a RID complex in a cell and to cells transfected with this vector. The vector comprises a nucleotide sequence encoding the RID α and RID β polypeptide components of the complex operably linked to a promoter capable of directing expression of the nucleotide sequence in the cell. A preferred vector consists of 231-10 (SEQ ID NO:2), which expresses functional polypeptides for all of the E3 genes other than *adp*.

30 Among the several advantages found to be achieved by the present invention, therefore, may be noted the provision of compositions and methods for inhibiting apoptosis of a cell expressing a death receptor; the provision of compositions and methods for promoting tissue transplant survival in patients; the provision of compositions and methods for treating patients suffering from an autoimmune disease and other disorders associated with

dysfunction of apoptosis regulation; and the provision of compositions and methods for promoting tumor destruction in cancer patients.

Brief Description of the Drawings

5 Figure 1 shows an alignment of the amino acid sequences of the death domains of the death receptor subfamily of TNFR proteins, with residues identical in more than 30% of sequences shaded black and residues conserved in more than 30% of sequences shaded in gray;

10 Figure 2 is a schematic representation of apoptosis mediated by death domain-containing members of the TNF receptor superfamily, with the death receptors Fas, TNFR1, TRAIL-R1, TRAIL-R2 and DR3 depicted by the bars on the extreme right and left sides of the figure, the ligands for these receptors indicated in parenthesis, and showing the association of the death receptors with intracellular proteins in the apoptotic signaling cascade at the bottom of the figure;

15 Figure 3 is a schematic representation of a preferred RID complex showing one mature 14.5K polypeptide having an O-glycosylated residue in the extracellular (or luminal) domain and an O-phosphorylated residue in the cytoplasmic domain, and two covalently-linked 10.4K polypeptides, one of which is an uncleaved, full-length form of 10.4K (10.4K-L) having two membrane-spanning regions (diagonal stripes) and the other a cleaved, short
20 form of 10.4K (10.4K-S) with only one transmembrane region;

 Figure 4 illustrates the amino acid sequences and various domains of preferred embodiments of the RID α and RID β polypeptides, showing in Fig. 4A-4B the long and short forms of the E3 10.4K polypeptides (RID α -L and RID α -S) from Ad serotype 2, Fig. 4C the pre-14.5K (RID β) polypeptide of Ad serotype 5, and in Fig. 4D the mature 14.5K (RID β)
25 polypeptide of Ad serotype 5, with the signal sequences and transmembrane domains underlined and the asterisks indicating sites for disulfide linkage in RID α or for O-phosphorylation in RID β ;

 Figure 5 is a schematic representation of a model for RID-induced internalization and degradation of Fas and TNFR1 death receptors, showing RID and the death receptor in the
30 plasma membrane, entry of RID and the death receptor into endosomes, transport of these endosomes to lysosomes where the death receptor is degraded, and recycling of RID in endosomes to the cell surface, where it can internalize another death receptor molecule;

 Figure 6 shows photographs of MCF7-Fas cells (Figs. 6A and 6B) infected with *rec700* Ad ("wild-type") or (Figs. 6C and 6D) transiently transfected with pMT2-RID α plus
35 pMT2-RID β which were then treated with an agonist monoclonal antibody to Fas and double-

stained for the adenovirus-encoded DNA binding protein (anti-ADP) (Fig. 6A) and for DNA 4, 6-diamidino-2-phenylindole (DAPI) (Fig. 6B) or double-stained for RID β (Fig. 6C) and DNA (Fig. 6D), with the photographs taken using a 100X Plan apo objective lens;

5 Figure 7 shows flow cytometry tracings of MCF7-Fas cells which were mock-infected (Fig. 7A) or infected with wild-type Ad (Ad5 and *rec700*) (Figs. 7B-7C) or with the indicated Ad E3 mutant (Figs. 7D-7H) and then incubated with antibodies to Fas (bold trace), transferrin receptor (dashed trace), or control IgG (light trace);

10 Figure 8 shows flow cytometry tracings of A549 cells which were mock-infected (Fig. 8B) or infected with wild-type Ad (*rec700*) (Fig. 8C) or with the indicated Ad E3 mutant (Figs. 7D-7H) and then incubated with antibodies to Fas (red trace), transferrin receptor (blue trace), or control IgG (black trace), with the cell pattern for mock-infected cells shown in Fig. 8A and R1 indicating the cells that were gated for the analysis;

15 Figure 9 shows photographs of mock-infected MCF7 cells (Fig. 9A) or MCF7-Fas cells mock-infected (Fig. 9B) or infected with the indicated viruses (Figs. 9C-9H) and then analyzed for Fas by immunofluorescence, with the speckled pattern in Figs. 9C, 9G, and 9H representing putative endosomes and lysosomes containing Fas;

20 Figure 10 shows an immunoblot of proteins extracted from MCF-7 Fas cells following mock-infection or infection with the indicated wild-type and mutant Ads and stained for Fas (Fig. 10A), transferrin receptor (Fig. 10B) or Ad E1A (Fig. 10C), with molecular weight markers indicated on the right;

25 Figure 11 shows photographs of COS7 cells transfected with expression plasmids for Fas and RID α (Fig. 11A, 11B), Fas and RID β (Fig. 11C, 11D), or Fas, RID α , and RID β (Fig. 11E-11H) and double-stained for RID α and Fas (Fig. 11A, 11B, 11E, 11F) or for RID β and Fas (Fig. 11C, 11D, 11G, 11H) with arrow in Figs. 11G and H indicate vesicles that appear to contain both RID β and Fas;

30 Figure 12 shows photographs of *rec700*-infected A549 cells double-stained for Fas and a lysosomal protein, LAMP1 and examined by confocal microscopy, with Fig. 12A showing cells labeled with rabbit anti-Fas antibody and fluorescein isothiocyanate (FITC), Fig. 12B showing cells labeled with mouse anti-LAMP-1 antibody and rhodamine isothiocyanate (RITC), Fig. 12C showing the combined images of Fig. 12A and 12 B, and Fig. 12D showing a perpendicular view of the image in Fig. 12C (arrows), 1 μ m thick, where green indicates Fas, red indicates LAMP-1 and yellow indicates colocalization of Fas and LAMP1 and the bar indicating a distance of 10 μ m;

Figures 13A-13C show photographs of immunofluorescence labeling of Fas in *rec700*-infected cells treated (Fig. 13A) or not treated (Fig. 13B) with bafilomycin A1 (Baf), or in *dI309* (RID⁻)-infected cells treated with Baf (Fig. 13C);

Figure 13D shows an immunoblot of proteins extracted from mock-, *rec700*- or *dI309*-infected cells treated (+) or not treated (-) with bafilomycin A1 (Baf) and stained for Fas, ERp72, or Ad protein E1B-19K;

Figure 13E shows the immunoblot of Fig. 13D following removal of antibody and restaining for transferrin receptor (TfR);

Figure 14 shows an immunoblot of proteins extracted from COS7 cells transfected with various combinations of plasmids expressing Fas, Shp-1, RID α or RID β as indicated by the "-" and "+" signs and stained for Fas, Erp72 or Shp-1 using appropriate antisera, with the arrows indicating two groupings of bands which correspond to differently glycosylated species of Fas;

Figure 15 shows an immunoblot of proteins extracted from COS7 cells transfected with various combinations of plasmids expressing Fas, chloramphenical acetyl-transferase (CAT), RID α or RID β as indicated by the "-" and "+" signs and stained for Fas, Erp72 or CAT using appropriate antisera, with the arrows indicating two groupings of bands which correspond to differently glycosylated species of Fas;

Figures 16A and 16B are graphs of the amount of lysis of mock-, *rec700*- or *dI7001*-infected Fas-positive mouse P815 cells by activated cytotoxic lymphocytes (CTL) from perforin (-/-) mice (Fig. 16A) or matched perforin (+/+) mice (Fig. 16B) at effector lymphocyte:target ratios of 60:1 (black bars), 20:1 (stippled bars), or 6:1 (open bars);

Figure 16C shows flow cytometry tracings of P815 cells infected with *rec700* (middle plot) or *dI7000* (right dark plot) and then stained for Fas, with the left plot showing the IgG control;

Figure 17 is a graph of the amount of lysis of mock- or Ad-infected Fas-positive human A549 cells by natural killer (NK) cells at NK:A549 cell ratios of 10:1 (black bar) and 5:1 (striped bar);

Figure 18 shows flow cytometry tracings of human HeLa cells mock-infected (green trace) or infected with *rec700* (red trace) or *dI712*, a mutant that overexpresses RID and E3-14.7K (blue trace) and then stained for TNFR1 (Fig. 18A) or Fas (Fig. 18B), with the percentage of cells that stained positive for TNFR1 or Fas indicated at the bottom;

Figure 19 shows flow cytometry tracings of human HeLa cells mock-infected (black trace) or infected with *rec700* (red trace), *dI753* (light blue trace), *dI764* (dark blue trace), *dI712* (green trace), *dI309* (pink trace) and then stained for TNFR1 (Fig. 19A) or Fas (Fig.

19B), with the genotype of each virus and the percentage of cells that stained positive for TNFR1 or Fas indicated at the bottom;

Figure 20 shows flow cytometry tracings of human HeLa cells mock-infected (black trace) or infected with the 231-10 vector, which expresses only the E3 proteins, and then stained for TNFR1 at 24 hr. p.i. (red trace) or 48 hr. p.i. (blue trace);

Figure 21 shows an immunoblot of TNFR1 extracted from A549 cells mock-infected or infected with rec700 in which cell surface proteins were labeled by incubation with biotin at the indicated hour p.i.;

Figure 22 shows an immunoblot of TNFR1 (Fig. 22A) and RID β (Fig. 22B) extracted from A549 cells mock-infected or infected with rec700 or the 231-10 vector in which cell surface proteins were labeled by incubation with biotin at the indicated hour p.i.;

Figure 23A shows an immunoblot of TNFR1 extracted from A549 cells mock-infected or infected with the indicated virus in which cell surface proteins were labeled by incubation with biotin at 26 h p.i.;

Figure 23B shows an immunoblot of Ad E1B-19K protein extracted from the same cells used in Fig. 23A;

Figure 24 shows a photograph of exposed skin and muscle of the hind flanks of a female C57Bl/6 mouse sacrificed 18 days after the flanks were subcutaneously injected with human cancer A549 cells infected with the 231-10 vector, with A549 tumors appearing as whitish-tan masses on each flank;

Figure 25 shows a closer view of the tumor on the right flank of the mouse in Fig. 24;

Figure 26 shows an immunoblot of proteins extracted from an A549 tumor grown in a mouse such as described in Fig. 24;

Figure 27 is a schematic illustration of the structure of the genome of the Ad 231-10 vector, with the black horizontal bar representing the backbone of the Ad5 genome, from which the E1 and E3 regions are deleted, as indicated by the triangles below the black bar, and containing an expression cassette with the CMV promoter controlling the E3 genes inserted into the deleted E1 region, as indicated by the triangle to the left, above the black bar, with the transcription unit oriented from right to left as indicated by the arrowhead and restriction endonuclease cleavage sites flanking the CMV-E3 cassette indicated;

Figure 28 illustrates the nucleotide sequence of the 231-10 genome with the numbering beginning with the first base-pair on the conventional left side of the Ad5 genome as shown in Fig. 27 and proceeding to the last base-pair at the right side of the genome;

Figure 29 shows an immunoblot of E3 RID β , 14.7K, and gp19K proteins expressed in A549 cells infected with the 231-10 vector and detected at the days p.i. indicated, with lane A

containing proteins extracted from 231-10-infected cells at 1 day p.i. following treatment with 1- β -D-arabinofuransylcytosine (araC) at 2 h p.i.; and

Figure 30 shows a photograph of A549 cells infected with the 231-10 vector and gp19K, RID β , and 14.7K proteins detected by indirect immunofluorescence.

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Detailed Description of the Invention

The present invention is based on the discovery that the Ad RID complex inhibits apoptosis mediated by death receptors, and in particular by Fas and TNFR1. Some of the molecular events involved in apoptosis induced through death receptors of the TNFR family are illustrated in Fig. 2. Fas (bar on the extreme right) is localized on the cell surface. When FasL engages Fas on the outside of the cell (top of Fig. 2), Fas associates with proteins within the cell (bottom of Fig. 2). First, Fas binds a protein named FADD through their corresponding death domains and then the Fas/FADD complex binds the protein named Caspase 8 through another region in FADD and Caspase 8 named the "death effector" domain. This binding activates the enzymatic activity of Caspase 8, an "initiator" caspase. Activated Caspase 8 cleaves other caspases (effector caspases), which then cleave other proteins, and apoptosis ensues. Apoptosis induced through TNFR is very similar, except that an additional protein, named TRADD, is involved. TNF engages TNFR1, causing it to bind TRADD through death domains in TNFR1 and TRADD (left part of Fig. 2). The TNFR1/TRADD complex then binds FADD through their death domains and this is followed by binding to Caspase 8, etc. TRAIL-R1, TRAIL-R2, and DR3 are believed to undergo a similar binding cascade to activate caspases, although the ligand that triggers apoptosis through DR3 is unknown.

RID inhibits apoptosis by means of an internalization and degradation mechanism common to all death receptors. As illustrated in Figure 2, RID shuttles the death receptor from the cell surface to lysosomes where the receptors are degraded. This model is supported in part by the fact that the RID complex has two motifs in its intracellular portion that are known to play a role in the internalization of some cell surface receptors and their transport to lysosomes. These motifs are a dileucine motif (LL), which is present in RID α , and a tyrosine-based motif in RID β , which is YXX ϕ , where Y is tyrosine, X is any amino acid, and ϕ is an aromatic or bulky hydrophobic amino acid such as phenylalanine, tyrosine, tryptophan and proline. It is believed that RID acts through the LL and YXX ϕ motifs to cause Fas or TNFR1 to be internalized into early/sorting endosomes. Again, acting through the LL and YXX ϕ motifs, RID mediates transport of the early endosomes to late endosomes and then to lysosomes where the receptors are degraded. RID then recycles back to the cell surface in

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endosomes where it repeats this process. Additional evidence supporting this model is as follows: (1) RID co-localizes with Fas on the cell surface as well as in vesicles; (2) degradation of Fas is inhibited by bafilomycin A1, an inhibitor of late endosome function; (3) the RID proteins are very stable, as indicated by pulse-chase experiments, whereas Fas is very unstable in the presence of RID; and (4) mutation of the LL motif severely reduces the function of RID, and conversion of the Y in the YXX ϕ motif abolishes the function of RID.

Because of their similar structures and common apoptotic pathway, it is believed that all death receptors of the TNFR family can be removed from the cell surface by RID via internalization into endosomes and subsequent degradation in lysozymes. Thus, RID will inhibit apoptosis mediated by any member of the TNFR death receptor family. As such, RID should be useful to promote survival of cells and tissues in the treatment of diseases such as degenerative diseases, immune disorders including autoimmune disorders, ischemic injury such as caused by myocardial infarction, stroke induced neuron death and reperfusion injury, alcohol-induced hepatitis, diseases caused by viral infection, such as AIDS and fulminant hepatitis, and cancer. RID is also useful in promoting survival of tissue transplants in transplant recipients.

Thus, in one embodiment the invention provides a method for inhibiting apoptosis of a cell comprising treating the cell with an effective amount of a Receptor Internalization and Degradation (RID) complex. Cells which can be treated by this method express one or more death receptors of the TNFR family, which includes Fas, TNFR1, DR3, TRAIL-R1, TRAIL-R2 and any subsequently discovered family member characterized by the presence of a death domain. Cells expressing a death receptor can be identified by methods known in the art, such as incubating the cells with one or more death receptor ligands followed by evaluating the cells for apoptosis, detecting death receptor molecules on the cell surface with an antibody against the death receptor, or detecting mRNA molecules that encode the death receptor. Cell death by apoptosis is readily recognizable and includes cytoplasmic and nuclear condensation, loss of membrane integrity and extensive fragmentation of chromosomal DNA, which forms a characteristic ladder when analyzed by gel electrophoresis. Vaux, D., *Proc. Natl. Acad. Sci* 90:786-789, 1993. Antibodies against the TNFR death receptors are either commercially available or can be readily prepared using standard techniques.

The RID complex used in the method comprises at least one of each of the following polypeptides: a RID α -L polypeptide, a RID α -S polypeptide, and a RID β polypeptide. RID α and RID β are synonymous with the 10.4K and 14.5K proteins, respectively, which are encoded by two genes in the Ad E3 region. The basic structures of these polypeptides in a membrane are illustrated in Fig. 3. RID α -L comprises a first transmembrane domain, which

is an uncleaved signal sequence, an extracellular domain, an internal transmembrane domain, and a cytoplasmic domain. RID α -S lacks the signal sequence and thus comprises the extracellular domain, the internal transmembrane domain and the cytoplasmic domain. RID β comprises an extracellular domain, which preferably lacks the signal sequence as shown in Fig. 4D, a transmembrane domain and a cytoplasmic domain. When the RID complex is localized in membrane structures and vesicles within the cell, the extracellular domain is located in the lumen of these membranes and vesicles.

In preferred embodiments, the RID α -S and RID α -L polypeptides are covalently joined by a disulfide bond between cysteine residues in their extracellular domains which correspond by alignment with the Cys₃₁ residue of the Ad2 10.4K protein (Fig. 4A). Also, RID β preferably has a mucin type O-linked oligosaccharide attached to one or more amino acids in the extracellular domain and/or is phosphorylated at one or two serines in the cytoplasmic domain. (See Krajcsi et al., *Virol.* 187:492-498, 1992; Krajcsi et al., *Virol.* 188:570-579, 1992.) The location of these residues in RID β polypeptides encoded by E3 genes of different Ad serotypes can be determined by alignment with the amino acid sequence for the 14.5K protein of Ad5, which is shown in Fig. 4C.

A RID complex made by Ad *in vivo* is believed to contain RID α -L, RID α -S and RID β (lacking the signal sequence) polypeptides in about a 1:1:1 ratio. However, it is possible that various ratios of these polypeptides will be functional or that in some cases different ratios will be required to provide a functional complex.

The amino acid sequences of the RID α -L, RID α - β and RID β polypeptides comprising the RID complex may be identical to those of naturally-occurring Ad RID α (10.4K) and RID β (14.5K) proteins from any Ad serotype or may comprise functional variants of such naturally-occurring sequences. As stated above, the genes encoding the RID α and RID β proteins are highly conserved among Ad serotypes. These genes are also conserved in Ads from some non-human species. Thus, it is believed that their encoded products should function very similar to the RID α and RID β polypeptides from Ad2 and Ad5, which were used in the experiments described herein. In addition, the invention includes the use of RID complexes in which the RID α -L, RID α -S, and RID β polypeptides comprise homologous amino acid sequences, i.e., encoded by the same Ad serotype, or that comprises heterologous sequences, i.e., encoded by two or more Ad serotypes. Thus, for example, a RID complex may comprise (1) a RID α -L polypeptide comprising the RID α -L amino acid sequence from Ad2, (2) a RID α -S polypeptide comprising the RID α -S amino acid sequence from Ad5, and (3) a RID β polypeptide comprising the RID β amino acid sequence from Ad9. Preferably, the RID complex comprises polypeptides whose amino acid sequences correspond

to serotypes from the same subgroup. More preferably, the RID complex comprises RID α -S and RID α -L polypeptides encoded by the RID α gene of Ad2 and a RID β polypeptide encoded by the RID β gene of Ad5.

5 A functional variant of a naturally-occurring RID α or RID β sequence contains one or more amino acid substitutions in that sequence which do not destroy the ability of the resulting polypeptide to function in a RID complex to inhibit apoptosis. Preferably, amino acid substitutions in functional variants are conservative amino acid substitutions, which refer to the interchangeability of residues having similar side chains. Conservatively substituted amino acids can be grouped according to the chemical properties of their side chains. For
10 example, one grouping of amino acids includes those amino acids have neutral and hydrophobic side chains (A, V, L, I, P, W, F, and M); another grouping is those amino acids having neutral and polar side chains (G, S, T, Y, C, N, and Q); another grouping is those amino acids having basic side chains (K, R, and H); another grouping is those amino acids having acidic side chains (D and E); another grouping is those amino acids having aliphatic
15 side chains (G, A, V, L, and I); another grouping is those amino acids having aliphatic-hydroxyl side chains (S and T); another grouping is those amino acids having amine-containing side chains (N, Q, K, R, and H); another grouping is those amino acids having aromatic side chains (F, Y, and W); and another grouping is those amino acids having sulfur-containing side chains (C and M). Preferred conservative amino acid substitutions groups are:
20 R-K; E-D, Y-F, L-M; V-I, and Q-H. In addition, conservative amino acid substitutions as used herein is intended to include substitutions which are present at corresponding positions in sequences from different Ad serotypes.

A functional variant as used herein can also include modified sequences in which one or more amino acids have been inserted, deleted, or replaced with a different amino acid or a
25 modified amino acid or unusual amino acid, as well as modifications such as glycosylation or phosphorylation so long as the polypeptide containing the modified sequence retains the biological activity of a RID α or RID β polypeptide. By retaining the biological activity, it is meant that the modified polypeptide can function to form a RID complex with anti-apoptotic activity.

30 In one embodiment, the cell is treated with the RID complex by administering to the cell a polynucleotide encoding the RID complex. The polynucleotide comprises a nucleotide sequence encoding a RID α polypeptide and a RID β polypeptide operably linked to a promoter that produces expression of the RID complex in the cell. In one variation of this embodiment, the polynucleotide can contain portions of the Ad E3 region in addition to that
35 portion encoding RID α and RID β . However, the polynucleotide predominantly expresses the

RID α and RID β proteins over any other Ad proteins. Alternatively, actions on cell apoptosis resulting from expression of the polynucleotide are predominantly due to the RID complex rather than any other protein expressed by the polynucleotide. The polynucleotide can comprise an expression plasmid, a retrovirus vector, an Ad vector, an adenovirus associated
5 vector (AAV) or other vector used in the art to deliver genes into cells. Alternatively, the polynucleotide can be administered to the cell by microinjection.

In embodiments where the cell being treated is in a patient, such as cells comprising a tissue transplant or a tissue involved in an autoimmune disorder, the polynucleotide encoding RID is administered to the patient. Any of the vectors discussed above can be used. It is also
10 contemplated that the RID complex be administered by coinfection with a replication-defective Ad expressing RID and another replication competent Ad that complements the replication defective virus to increase the expression of RID in the infected cells.

Preferably, the polynucleotide is selectively delivered to target cells within the patient so as not to affect apoptosis in other tissues. Targeted delivery of the polynucleotide can be
15 done for example by using delivery vehicles such as polycations, liposomes or viral vectors containing targeting moieties that recognizes and binds a specific marker on the target cell. Such methods are known in the art, see, e.g., U.S. Patent No. 5,635,383. Another targeted delivery approach uses viral vectors that can only replicate in specific cell types which is accomplished by placing the viral genes necessary for replication under the transcriptional
20 control of a response element for a transcription factor that is only active in the target cell. See, e.g., U.S. Patent No. 5,698,443.

In other embodiments of the invention, the cell is treated by administering to the cell a composition comprising a RID complex. The RID complex for use in such embodiments can be prepared by a variety of means. For example, the RID complex can be isolated from
25 the membranes of Ad-infected cells or cells transfected with a nucleotide sequence encoding the RID α and RID β polypeptides. Alternatively, the polypeptide components of the complex can be expressed in separate cell cultures, extracted into an appropriate buffer and mixed *in vitro*. RID α and RID β polypeptides can also be chemically synthesized and mixed to form the complex. The RID complex can then be tested for the ability to inhibit apoptosis of a cell
30 expressing a death receptor as described herein for Fas and TNFR1.

Preferably, the RID complex is administered with a carrier that facilitates delivery of the RID complex into the cell, such as liposomes. Where the RID complex is being administered to a patient, the liposomes can have targeting moieties exposed on the surface such as antibodies, ligands or receptors to specific cell surface molecules to limit delivery of
35 RID to targeted cells. Liposome drug delivery is known in the art (see, e.g., Amselem et al.,

Chem. Phys. Lipid 64:219-237, 1993). Alternatively, one or more of the polypeptides of the complex can be modified to include a specific transit peptide that is capable of delivering the peptide into the cytoplasm of a cell or the complex can be delivered directly into a cell by microinjection.

5 Compositions comprising a RID complex can be administered by any suitable route known in the art including, for example, intravenous, subcutaneous, intramuscular, transdermal, intrathecal or intracerebral or administration to cells in *ex vivo* treatment protocols. Administration can be either rapid as by injection or over a period of time as by slow infusion or administration of slow release formulation. For treating tissues in the central
10 nervous system, administration can be by injection or infusion into the cerebrospinal fluid (CSF). When it is intended that the RID complex be administered to cells in the central nervous system, administration can be with one or more agents capable of promoting penetration of the protein complex across the blood-brain barrier.

 The RID complex can also be linked or conjugated with agents that provide desirable
15 pharmaceutical or pharmacodynamic properties, including for example, substances known in the art to promote penetration or transport across the blood-brain barrier such as an antibody to the transferrin receptor (Friden et al., *Science* 259:373-377, 1993), a polymer such as polyethylene glycol to obtain desirable properties of solubility, stability, half-life and other pharmaceutically advantageous properties Davis et al. *Enzyme Eng* 4:169-73, 1978; Burnham,
20 *Am J Hosp Pharm* 51:210-218, 1994).

 For nonparental administration, the compositions can also include absorption enhancers which increase the pore size of the mucosal membrane. Such absorption enhancers include sodium deoxycholate, sodium glycocholate, dimethyl- β -cyclodextrin, lauroyl-1-lysophosphatidylcholine and other substances having structural similarities to the
25 phospholipid domains of the mucosal membrane.

 The compositions are usually employed in the form of pharmaceutical preparations. Such preparations are made in a manner well known in the pharmaceutical art. One preferred preparation utilizes a vehicle of physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers such as physiological concentrations of other non-toxic
30 salts, five percent aqueous glucose solution, sterile water or the like may also be used. It may also be desirable that a suitable buffer be present in the composition. Such solutions can, if desired, be lyophilized and stored in a sterile ampoule ready for reconstitution by the addition of sterile water for ready injection. The primary solvent can be aqueous or alternatively non-aqueous.

35 The carrier can also contain other pharmaceutically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate

of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmaceutically-acceptable excipients for modifying or maintaining release or absorption or penetration across the blood-brain barrier. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral administration in either unit dosage or multi-dose form or for direct infusion by continuous or periodic infusion.

It is also contemplated that certain formulations comprising the RID complex are to be administered orally. Such formulations are preferably encapsulated and formulated with suitable carriers in solid dosage forms. Some examples of suitable carriers, excipients, and diluents include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, gelatin, syrup, methyl cellulose, methyl- and propylhydroxybenzoates, talc, magnesium, stearate, water, mineral oil, and the like. The formulations can additionally include lubricating agents, wetting agents, emulsifying and suspending agents, preserving agents, sweetening agents or flavoring agents. The compositions may be formulated so as to provide rapid, sustained, or delayed release of the active ingredients after administration to the patient by employing procedures well known in the art. The formulations can also contain substances that diminish proteolytic degradation and/or substances which promote absorption such as, for example, surface active agents.

The RID complex is administered to patients in an amount effective to inhibit apoptosis of target cells within the patient. The specific dose is calculated according to the approximate body weight or body surface area of the patient or the volume of body space to be occupied. The dose will also be calculated dependent upon the particular route of administration selected. Further refinement of the calculations necessary to determine the appropriate dosage for treatment is routinely made by those of ordinary skill in the art. Such calculations can be made without undue experimentation by one skilled in the art in light of the activity disclosed herein in cell death assays. Exact dosages are determined in conjunction with standard dose-response studies. It will be understood that the amount of the composition actually administered will be determined by a practitioner, in the light of the relevant circumstances including the condition or conditions to be treated, the choice of composition to be administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the chosen route of administration. Dose administration can be repeated depending upon the pharmacokinetic parameters of the dosage formulation and the route of administration used.

The compositions and methods of the invention are contemplated for use in promoting survival of tissue transplants. For example, the tissue can be treated *in vitro* with the RID complex and the treated tissue then introduced into the transplant. In addition,

previously transplanted tissues can be treated with RID by administering the RID complex to the transplant recipient. In either scenario, it is contemplated that the RID complex can be administered as a protein formulation or as a polynucleotide expressing the complex.

In another embodiment, the RID complex is used to promote the survival of leukocytes in cancer patients. The leukocytes can be treated *in vivo* by administering to the patient a polynucleotide expressing RID or a composition containing the RID complex. Preferably, the polynucleotide or RID complex is targeted to the leukocytes by one of the targeting methods discussed above. For example, cytotoxic T cells could be targeted by using an antibody against the CD8 marker and natural killer cells targeted by use of an antibody against the CD16 marker. Alternatively, the leukocytes can be removed from the patient, treated with the RID complex *ex vivo*, and the treated leukocytes then returned to the patient.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

Example 1

This example illustrates inhibition of Fas-mediated apoptosis by adenovirus E1B and E3 proteins.

Human breast adenocarcinoma cells expressing Fas (MCF7-Fas) (Jäättelä et al., *Oncogene* 10:2297-2305, 1995) were infected with *rec700* or with an adenovirus mutant lacking expression of one or more of the RID α , RID β , E3-14.7K and E1B-19K proteins. *rec700* is an Ad5-Ad2-Ad5 "wild-type" recombinant whose genome consists of the Ad5 *EcoRI* A (map positions 0 to 76), Ad2 *EcoRI* D (map positions 76 to 83), and Ad5 *EcoRI* B (map positions 83 to 100) fragments (Wold et al., *Virol.* 148:168-188, 1986). *rec700* is the parental virus of E3 mutants with 700 or 7000 numbers. The infected cells were treated with a monoclonal antibody to Fas, CH-11, which acts as an agonist of Fas and induces apoptosis. The cells were then fixed and stained for DNA and for the adenovirus DNA binding protein (DBP). Experimental details are provided in the footnote to Table 1.

Examples of apoptotic and non-apoptotic nuclei in *rec700*-infected cells are shown in Figs. 6A and 6B. Most cells were infected as indicated by the speckled staining of DBP in the nucleus (Fig. 6A), and these nuclei were non-apoptotic (Fig. 6B). Two uninfected cells were apoptotic (arrows in Figs. 6A and 6B) as evidenced by the presence of shrunken and irregular nuclei with condensed DNA that often fluoresced very brightly above the plane of

focus for non-apoptotic nuclei. The percentage of apoptotic and non-apoptotic nuclei was scored in *rec700*- or mutant-infected cells staining for DBP and the quantitative results are shown in Table 1 below.

5 Table 1. Fas Agonist-induced Apoptosis in MCF7-Fas Cells Infected with Ad Mutants¹

Virus Mutant	Ad DNA Binding Protein-Positive Cells ²	
	Apoptotic	Non-apoptotic
<i>rec700</i> (wild type)	0.1 ³	99.9 ³
<i>pm760</i> (E1B-19K ⁻ , RID ⁻)	0.7	99.3
<i>dl309</i> (E1B-19K ⁻ , RID ⁻)	0.1	99.9
<i>dl748</i> (E1B-19K ⁻ , RID ⁻)	0.6	99.4
<i>dl764</i> (E1B-19K ⁻ , RID ⁻)	0.2	99.8
<i>lp5</i> (E1B-19K ⁻ , RID ⁻)	9.9	90.2
<i>dl250</i> (E1B-19K ⁻ , RID ⁻)	10.4	89.6
<i>dl111</i> (E1B-19K ⁻ , RID ⁻)	87.2	12.8
<i>dl118</i> (E1B-19K ⁻ , RID ⁻)	94.1	5.9

¹MCF7-Fas cells were infected with 250 PFU per cell of virus except for *lp5*, *dl250*, *dl111*, and *dl118* where 10 PFU per cell was used. At 21 h post-infection (p.i.), cells were treated for 22 h with the CH-11 agonist mAb to Fas (200 ng/ml) (Panvera, Madison, WI) plus cycloheximide (25 µg/ml). Cells were fixed and stained for the Ad DNA binding protein (DBP) using a rabbit antiserum (obtained from Maurice Green, St. Louis University) and goat anti-rabbit IgG (fluorescein conjugate) and for DNA using 4, 6-diamidino-2-phenylindole (DAPI). Typical apoptotic and non-apoptotic nuclei are shown in Fig. 6B, which is from the same experiment. Nuclei of *dl111*- or *dl118*-infected cells not treated with Fas agonist were not apoptotic (not shown), indicating that the apoptosis observed was not due to the *cyt deg* phenotype of E1B-19K-negative mutants (Subramanian et al., *J. Virol.* 52:336-343, 1984).

20 ²At least 1000 DBP-positive cells were counted per sample.

³Percent of apoptotic and non-apoptotic nuclei in cells staining for DBP.

In cells infected with *rec700* or mutant *pm760*, which expresses both E1B-19K and RID, very few nuclei were apoptotic. Cells infected with mutants expressing E1B-19K but lacking RID α and E3-14.7K (*d1748*), or lacking RID β (*d1764*), or lacking each of RID α , RID β , and E3-14.7K (*d1309*) also had very few apoptotic nuclei. However, only about 10% of cells infected with *lp5* and *d1250*, which lack E1B-19K but express RID, had apoptotic nuclei, while about 90% of the nuclei were apoptotic in cells infected with *d1111* and *d1118*, which lack expression of RID α , RID β , E3 14.7 K and E1B-19K. These results indicate that adenovirus has two proteins that independently inhibit Fas-induced apoptosis, RID and/or E3-14.7K in the E3 transcription unit and E1B-19K in the E1B transcription unit. This result observed with E1B-19K is consistent with an earlier report (Hashimoto, S., et al., *Int. Immunol.* 3:343-351, 1991. Data below show that RID inhibits Fas-induced apoptosis.

Example 2

This example illustrates that the RID complex is sufficient to inhibit apoptosis. To address whether RID is sufficient to inhibit Fas-induced apoptosis, plasmids expressing RID α or RID β from the Ad major late promoter plus SV40 enhancer were prepared by cloning the gene for RID α or RID β into the pMT2 vector (Mazzarella, R. A. & Green, M. J. *Biol. Chem.* 262: 8875-8883, 1987) to generate pMT2-RID α and pMT2-RID β . MCF7-Fas cells were transiently transfected with pMT2-RID α plus pMT2-RID β , pMT2-RID β alone, or pMT2 alone (2.5 μ g for each plasmid). After 38 h, cells were treated for 9 h with the CH-11 agonist mAb to Fas (500 ng/ml) plus cycloheximide (25 μ g/ml), fixed in methanol with DAPI, and stained for RID β using the rabbit P118-132 anti-peptide antiserum (Tollefson et al., *Virology* 175:19-29, 1990).

Examples of apoptotic and non-apoptotic nuclei in the cells co-transfected with pMT2-RID α and pMT2-RID β are shown in Figs. 6C and 6D. The cell transfected with RID α plus RID β (arrow in Fig. 6C) was non apoptotic (arrow in Fig. 6D). RID β -negative cells usually had apoptotic nuclei (most cells in Fig. 6D). Of 2000 cells counted in random fields, 173 RID β -positive cells were seen, and only 26% of these had apoptotic nuclei. In the transfection with RID β alone, and with 2000 cells counted, 101 RID β -positive cells were seen, 80% of which had apoptotic nuclei. With pMT2 alone, 62% of the total nuclei were apoptotic. These results indicate that RID (i.e. RID α plus RID β), but not RID β alone, is sufficient to inhibit Fas-induced apoptosis.

Example 3

This example illustrates that RID down-regulates Fas from the cell-surface of adenovirus-infected human breast carcinoma cells.

To investigate how RID inhibits apoptosis, MCF7-Fas cells were infected with
5 adenovirus serotype 5 (Ad5), *rec700*, or an Ad mutant lacking expression of one or more of
RID α , RID β , and E3-14.7K proteins. At 28 h p.i., cells were detached using 0.025% EDTA,
then resuspended in FACS buffer (1X PBS, 2% FBS). Approximately 1×10^6 cells were
pelleted and resuspended in 50 μ l FACS buffer containing antibodies against human Fas
(UB2 IgG mAb) (Panvera) (10 μ g/ml), the human transferrin receptor
10 (Boehringer/Mannheim, Indianapolis, IN) (2.5 μ g/ml) and purified mouse IgGy (PharMingen,
San Diego, CA) (5 μ g/ml) as an iso-type control. In common with Fas, the transferrin
receptor is a cell surface receptor. Cells were incubated with the primary antibodies, washed
with cold FACS buffer, incubated with 20 μ g/ml of goat anti-mouse FITC-conjugated
antibody (ICN), washed, then analyzed on a FACScaliber flow cytometer (Becton Dickinson,
15 Mountain View, CA). The data were analyzed with Cell Quest software (Becton Dickinson)
and are shown in Figure 7.

Nearly all Fas (bold trace in Fig. 7) was cleared from cells infected with Ad5 or
rec700 (Figs. 7B, 7C). Transferrin receptor (dashed trace) was not affected. Fas was not
cleared from cells infected with mutants lacking RID α and/or RID β , namely *dl309* (lacks
20 RID α , RID β , E3-14.7K) (Fig. 7D), *dl748* (lacks RID α) (Fig. 7E), and *dl764* (lacks RID β)
(Fig. 7F). Fas was down-regulated by *dl758* (RID-positive, lacks E3-14.7K) (Fig. 7G) and
pm760 (overexpresses RID α and RID β) (Fig. 7H). These results indicate that RID (i.e. RID α
and RID β) is necessary to clear Fas from the surface of Ad-infected MCF7-Fas cells. Other
Ad proteins, including E3-14.7K and E1B-19K, are not required.

25

Example 4

This example illustrates that RID down-regulates Fas from the cell-surface of adenovirus-infected human lung adenocarcinoma cells.

To determine if RID can remove Fas from the surface of other cell types, the human
30 A549 cell line was examined. A549 cells are derived from a human lung adenocarcinoma.
A549 cells were mock-infected or infected with *rec700*. At 26 h p.i., cells were suspended in
FACS buffer containing mouse IgGy, anti-human-Fas UB2 IgG monoclonal antibody
(Panvera), or antibody against the human transferrin receptor (Boehringer/Mannheim),
incubated with goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody, and

analyzed on a FACScaliber flow cytometer using Cell Quest software (Becton Dickinson). The results are shown in Fig. 8.

With mock-infected cells (Fig. 8B), there was strong staining for both Fas (the red trace in Fig. 8) and transferrin receptor (the blue trace in Fig. 8). With *rec700* or *pm760*, a virus mutant that overexpresses RID (i.e., RID α plus RID β) and underexpresses other Ad E3 proteins, Fas was completely cleared from the cell surface whereas the transferrin receptor was not affected (Figs. 8C, 8H). With three virus mutants that lack both RID α and RID β (*dl309*), RID β only (*dl764*), or RID α (*dl748*), Fas was not cleared from the cell surface (Fig. 8, Panels E, F, and G). With *dl758*, a mutant that lacks only E3-14.7K and that expresses RID α and RID β , Fas was down-regulated to the same extent as with *rec700* and *pm760*. Therefore, the E3-14.7K protein is not required to down-regulate cell surface Fas. Recently, RID was reported to clear Fas from the cell surface in two other human cell lines, HT-29.14S and ME-180 (Shisler et al., *J. Virol.* 71:8299-8306, 1997). These results have been confirmed with HT-29.14S and ME-180 cells (data not shown). Thus, RID stimulates the removal of Fas from the cell surface of at least four different cell types, MCF7-Fas, A549, HT-29.14S, and ME-180 cells.

Example 5

This example illustrates that Fas molecules removed from the cell surface by RID are internalized into vesicles and then degraded in lysosomes.

Many receptors are internalized into endosomes. Accordingly, MCF7-Fas cells were mock-infected or infected with *rec700* or with an E3 Ad mutant. MCF7 cells were mock-infected as a control. At 19 h p.i., cells were fixed in methanol and stained for Fas using the ZB4 mAb (Panvera) and goat anti-mouse IgG (Texas red conjugate). The results are shown in Figure 9.

Fas was not detected in mock-infected parental MCF7 cells (Fig. 9A), but was readily apparent on the surface of MCF7-Fas cells (Fig. 9B). In cells infected with *rec700*, Fas was in numerous vesicles and there was no cell surface staining (Fig. 9C). These vesicles are likely to be endosomes and lysosomes containing Fas. These vesicles were not observed with *dl309*, *dl748*, or *dl764* (lack RID α and/or RID β), whereas in each case, strong Fas staining was apparent at the plasma membrane (Figs 9D-9F). Vesicles staining for Fas were seen with *dl758* and *pm760*, both of which express RID (Figs. 9G, 9H).

Some receptor types internalized into endosomes are targeted to lysosomes where they are degraded. To determine whether Fas was degraded in Ad-infected cells expressing RID, MCF7-Fas cells were mock-infected or infected with wild-type Ad or an E3 mutant

lacking expression of one or more of RID α , RID β , and 14.7K proteins, then at 27 h p.i. proteins were extracted, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto an Immobilon-P membrane. After blocking, membranes were incubated with rabbit anti-Fas antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-transferrin receptor mAb OKT9 (ATCC), or mouse anti-E1A mAb M73. Membranes were incubated with the appropriate peroxidase-conjugated secondary antibody (ICN). Proteins were detected with ECL reagents (Amersham Life Sciences, Arlington Heights, IL) and the results are shown in Fig. 10.

Fas was degraded in cells infected with viruses that express both RID α and RID β (Ad5, Ad2, *rec700*, *dl758*, *pm760*) (Fig. 10A). Transferrin receptor was not degraded in these same extracts (Fig. 10B). Fas expression was actually stimulated in cells infected with mutants that lack RID α and/or RID β (Fig. 10A, compare Mock with *dl309*, *dl748*, and *dl764*). The Ad-coded E1A proteins were expressed at similar levels (Fig. 10C), indicating that all infections were equivalent. These and the above results establish that RID (i.e. RID α and RID β) functions in the internalization of Fas into putative endosomes, the degradation of Fas, and the inhibition of Fas-induced apoptosis.

RID has been reported to stimulate the internalization of EGFR into vesicles and its degradation in lysosomes (Carlin et al., *Cell* 57:135-144, 1989; Tollefson et al., *J. Virol.* 65:3095-3105, 1991). When the epidermal growth factor receptor (EGFR) interacts with its ligand, EGF, EGFR is internalized into early endosomes which are transported to late endosomes which fuse with lysosomes, where EGFR is degraded. This process results in attenuation of signal transduction through EGFR. Many receptors are degraded by the endosome-lysosome pathway in response to ligand. To determine if RID-induced degradation of Fas is occurring through this pathway, the following experiments were performed.

The first experiment, which was described in the copending provisional application, examined Fas localization in COS cells transiently co-transfected with combinations of expression plasmids for Fas, RID α and RID β . The following plasmids were used, the pMT2-RID α and pMT2-RID β plasmid vectors described in Example 2, and pcDNA3-Fas, which expresses Fas from the human cytomegalovirus promoter (CMV). COS7 cells were transfected (Mazzarella, R. A. & Green, M. *J. Biol. Chem.* 262:8875-8883, 1987) with 1 μ g each of pMT2-RID α plus pcDNA3-Fas, pMT2-RID β plus pcDNA3-Fas, or pMT2-RID α , pMT2-RID β , and pcDNA3-Fas. After 30 h, cells were fixed in methanol with DAPI and stained for Fas using the ZB4 mAb, for RID α using the rabbit P77-91 anti-peptide antiserum, or for RID β using the rabbit P118-132 anti-peptide antiserum (Tollefson et al., *J. Virol.*

64:794-801, 1990; Tollefson et al., *Virology* 175:19-29, 1990). The results are shown in Figure 11.

With cells co-transfected with expression plasmids for RID α plus Fas, or RID β plus Fas, Fas was localized on the cell surface (Fig. 11B, 11D). In contrast, with cells triple-
5 transfected with expression plasmids for RID α , RID β , and Fas, Fas was in vesicles rather than the cell surface (Fig. 11F, 11H). RID β staining was typical of the endoplasmic reticulum (ER) and plasma membrane, a probable site of RID action (Stewart et al., *J. Virol.* 69:172-181, 1995); many vesicles containing RID β appeared to co-localize with vesicles containing Fas (arrows in Fig. 11G and 11H). Distribution to the ER was also characteristic of RID α
10 (Fig. 11E), and in some cells the plasma membrane was stained (not shown). RID α did not co-localize with Fas-containing vesicles. Thus, RID (i.e. RID α plus RID β) is sufficient to internalize Fas into vesicles.

In a second experiment, Fas localization was examined in Ad-infected cells. Human A549 cells were infected with rec700 fixed using 3.7% paraformaldehyde followed by
15 methanol/DAPI (4,6-diamidino-2-phenylindole). Cells were double-stained for Fas and LAMP1, which is a lysosomal protein (Carlsson et al., *J. Biol. Chem.* 15:18911-18919, 1988), using a rabbit anti-Fas antibody (Santa Cruz Biotechnology) and the BB6 mouse anti-human-LAMP-1 monoclonal antibody (Carlsson et al., *supra*), followed by goat anti-rabbit IgG-FITC and goat anti-mouse IgG-RITC (rhodamine isothiocyanate) (Cappel ICN). Cells
20 were examined using a Zeiss LSM 410 scanning laser confocal microscope with LSM 410 software. The results are shown in Figure 12.

Green, red, and yellow vesicles contain Fas (Fig. 12A), LAMP1 (Fig. 12B), or both Fas and LAMP1 (Fig. 12C, 12D), respectively. The many yellow vesicles establish that Fas co-localizes with LAMP1 in lysosomes. The Fas-containing green vesicles may be
25 endosomes. Similar results were obtained with another lysosomal protein, CD63 (data not shown).

To obtain additional evidence supporting the involvement of the endosome-lysosome pathway in RID-induced Fas degradation in Ad-infected cells, the effect of Bafilomycin A1 (Baf) treatment was investigated. Baf specifically inhibits the vacuolar-type H⁺-ATPase,
30 preventing vesicle acidification and trafficking of receptors from late endosomes to lysosomes (Yoshimori et al., *J. Biol. Chem.* 266:17707-17712, 1991; van Weert et al., *J. Cell. Biol.* 130:821-834, 1995). A549 cells were mock-infected or infected with rec700 or d1309 (lacks RID). At 13 h after infection, cells were treated with Baf (0.1 μ M) for 12 h and then immunostained for Fas. In a separate experiment, cells were treated with Baf at 6 h after

infection and processed for immunoblot analysis 18 h later. The results are shown in Figure 13.

When wild-type Ad-infected cells were treated with Baf, Fas was cleared from the cell surface but it accumulated in vesicles (Fig. 13A) rather than being degraded as in untreated cells (Fig. 13B). Baf did not affect cell surface Fas in cells infected with a mutant lacking RID (*d/309*) (Fig. 13C). Immunoblot analysis of proteins extracted from these cells indicated that Baf blocked the degradation of Fas in wild-type Ad-infected cells (Fig. 13D). Baf did not affect the abundance of Fas in mock-infected cells or in cells infected with the RID-minus mutant. Neither virus infection nor Baf affected the abundance of Erp72 (Fig. 13D), a cellular protein localized in the endoplasmic reticulum (Mazzarella et al., 1990). Also, neither virus infection nor Baf significantly affected the level of another cellular protein, the transferrin receptor (Fig. 13E). The infections were equivalent as indicated by the E1B-19K levels of the Ad-encoded protein (Fig. 13D). These confocal microscopy and Baf data provide strong evidence that RID causes Fas to be degraded in lysosomes in Ad-infected cells.

Example 6

This example illustrates that the RID proteins are sufficient to promote the degradation of Fas.

COS cells were transiently transfected with different combinations of pMT2-RID α , MT2-RID β , pcDNA3-Fas, and pBUC-Shp-1, which expresses a mammalian cell protein named Shp-1. At 36 h post-transfection, cells were treated with cycloheximide (25 μ g/ml) for 12 h and at 48 h post-transfection, proteins were extracted and analyzed for Fas, Shp-1, or ERp72 by immunoblot using rabbit antisera to Fas (Santa Cruz), Erp72 (Mazzarella et al., 1990), or Shp-1 (Plas et al., 1996) (Tollefson et al., Nature 392:726-730 (1998)). The results are shown in Fig. 14.

In cells transfected with pcDNA3-Fas and/or pBUC-Shp-1, expression of Fas and/or Shp-1 proteins was readily detected by immunoblot (Fig. 14, lanes b-d). For Fas, two groupings of bands were detected (indicated by the arrows), which represent differentially glycosylated species of Fas. The anti-Fas antibody also reacted with an unknown cellular protein that migrated between the two sets of Fas protein bands. When pMT2-RID α or pMT2-RID β were co-transfected with pcDNA3-Fas and pBUC-Shp-1, there was a marginal decrease in Fas and Shp-1 (Fig. 14, lanes e and f). However, when both pMT2-RID α and pMT2-RID β were co-transfected with pcDNA3-Fas and pBUC-Shp-1, the Fas bands were reduced to nearly undetectable levels, whereas the Shp-1 band was only marginally decreased

(Fig. 14, lane g). The levels of the endogenous cellular protein, Erp72, were equivalent in all of the transfected cells. These results indicate that the RID complex (i.e. RID α plus RID β), but not RID α or RID β alone, is sufficient to induce degradation of Fas.

5 A similar experiment was conducted except that cells were transfected with the pcDNA3.1-CAT (InVitrogen, Carlsbad, CA) plasmid expressing chloramphenicol acetyl transferase (CAT) instead of pBUC-Shp-1. Since CAT is a bacterial protein, it is not possible for RID to have evolved in Ad to exert a specific biological effect on CAT. Expression of this protein was detected by immunoblot using anti-CAT antiserum obtained from 5 prime-3 prime. The results of the experiment were similar to those with Shp-1, i.e. Fas was greatly
10 reduced in the presence of RID, whereas CAT was only marginally affected (Fig. 15).

These experiments demonstrate that the RID complex is sufficient to induce the internalization of cell-surface Fas into vesicles, presumably endosomes and lysosomes, to induce degradation of Fas, presumably in lysosomes, and to inhibit apoptosis triggered by an anti-Fas agonist monoclonal antibody.

15

Example 7

This example illustrates that RID inhibits killing of Ad-infected cells by natural killer cells and cytotoxic lymphocytes.

20 Natural killer (NK) cells and cytotoxic T-lymphocytes (CTL) play an important role in the destruction of virus-infected cells during the early innate phase and the late immune-specific stages, respectively, of the host anti-viral response. Both NK and CTL kill targets via two major pathways. In one major pathway, perforin generates holes in the target and granzymes are introduced to induce apoptosis of the target cell. In another major pathway, Fas ligand on the surface of the CTL engages Fas on the target cell and induces apoptosis
25 through activation of the pro-apoptotic caspases. CTL can also kill cells through a third minor pathway, in which TNF expressed on the surface of CTL (or secreted by CTL) engages TNFR1 on targets and induces apoptosis via the caspases. In cell culture, TNF-mediated killing by CTL is observable in long term (> 24 h) killing assays. To investigate whether RID inhibits NK- and CTL-killing through Fas, the following experiments were conducted.

30 In the first experiment, which was described in the copending provisional application, the effect of Ad proteins on CTL-killing was assessed by performing a short-term CD3-dependent redirected cell assay (Azuma et al., *J. Exp. Med.* 175:353-360, 1992), using lymphocytes from perforin (-/-) mice (Kagi et al., *Science* 265:528-530, 1994) and from wild-type perforin (+/+) C57BL/6J mice acutely infected with influenza virus. Influenza virus
35 enhances the expression of Fas ligand in activated lymphocytes (Clark et al., *Immunol. Rev.* 146:33-44, 1995). In brief, mice were primed by intranasal infection of 50 HAU of HkX31

influenza A virus (Topham et al., *J. Virol.* 70:1288-1291, 1996; Tripp et al., *J. Immunol.* 154:6013-6021, 1995). CTL were isolated from the spleens of the infected mice, irradiated, and effector CTL generated by secondary *in vitro* re-stimulation. These CTL were further activated by incubation with the 145-2C11 anti-CD3 ϵ mAb for 30 min on ice. Mouse Fas and Fc receptor-positive P815 cells (1×10^6) were mock-infected or infected with 1000 PFU per cell of *rec700* or *dI7001* and labeled overnight with 100 μ Ci of Na₂⁵¹CrO₄. These ⁵¹Cr-labeled P815 target cells were washed, resuspended in DME, and then incubated with the activated anti-CD3 ϵ -treated CTL using effector lymphocyte:target ratios of 60:1, 20:1 or 6:1. Cell lysis was determined 6 h later from a standard ⁵¹Cr release assay and the results are shown in Figs. 16A and 16B. The presence of Fas on the surface of P815 cells infected with *rec700* or *dI7000* was also examined by flow cytometry and the results are shown in Fig. 16C.

The perforin (-/-) CTL lysed mock-infected P815 cells efficiently (Fig. 16A). Lysis was inhibited by *rec700* but not by *dI7001* (lacks all E3 genes). Since the mice lack perforin, it follows that the CTL were killing the mock- and mutant-infected cells through the Fas pathway and that the E3 region is required to inhibit killing through this pathway. The CTL from perforin (+/+) mice killed mock-, *rec700*-, or *dI7001*-infected P815 cells with similar high efficiency (Fig. 16B). Cell surface Fas was diminished on P815 cells infected with *rec700* but not with *dI7000* (lacks all E3 genes except for E3-14.7K) (Fig. 16C). These results indicate that E3 proteins expressed by *rec700* but not *dI7000*, presumably RID, inhibit CTL killing through the Fas pathway by down-regulating Fas from the cell surface.

A second experiment was conducted to investigate the role of RID in inhibiting killing of Ad-infected cells by NK cells. Human A549 cells were mock-infected or infected with *rec700* (wild-type Ad) or *dI764*, a virus mutant that lacks only RID β and then labeled with 100 μ Ci of Na₂⁵¹CrO₄. These ⁵¹Cr-labeled A549 target cells were washed, resuspended in DME, and then incubated with a semi-permanent line of human NK cells. After 24 h, cell lysis was measured based on release of ⁵¹Cr from the cells as described elsewhere (Tollefson et al., *Nature* 392:726-730 (1998)) and the results are shown in Figure 17.

Mock-infected cells were lysed efficiently at NK:A549 cell ratios of 10:1 and 5:1 (Fig. 17). This lysis was dramatically inhibited by infection with *rec700*, but it was only marginally reduced by infection with *dI764* (Fig. 17). Since the only protein not expressed by *dI764* is RID β , it is believed that RID is required to inhibit killing of Ad-infected cells by NK cells. Most likely RID inhibits killing by NK cells by blocking the Fas pathway. However, a RID effect on the perforin-granzyme pathway cannot be excluded.

In summary, RID inhibits killing of Ad-infected cells by NK cells and by CTL. Thus, RID should protect infected cells from attack by killer cells that are active in both the early

innate phase and the late immune-specific phase of the anti-viral immune response. Similarly, transplanted cells and tissues are destroyed by NK cells and CTL. Therefore, RID should be useful to inhibit killing of transplanted cells or tissues by NK cells and CTL.

5

Example 8

This example illustrates that RID is required and probably sufficient to remove the TNFR1 from the cell surface.

Human HeLa cells were mock-infected or infected with 50 PFU/cell of *rec700* (wild-type) or *dl712*, which is a *rec700*-derived mutant with a deletion in the *adp* gene in the E3 region that results in overexpression of both RID (i.e. RID α and RID β) and E3-14.7K, and only trace amounts of other E3 proteins (Tollefson et al., *J. Virol.* 64:794-801, 1990; Tollefson et al., *Virol.* 175:19-29, 190; Gooding et al., *Cell* 53:341-346, 1988). At 26 h p.i., cells were analyzed by flow cytometry (Tollefson et al., *Nature* 392:726-730 (1998)) using the B/O:2/18/91 rabbit antiserum against TNFR1 (obtained from Immunex Corp.) and PE-conjugated goat anti-rabbit IgG (Caltag). Fas was detected in the same experiment using supernatants from the M38 anti-Fas hybridoma cell line (obtained from the American Type Culture Collection) and FITC-conjugated goat anti-mouse IgG. The results are shown in Figure 18.

As shown in Fig. 18A, TNFR1 was removed from the surface of most cells infected with *rec700* (red trace) or *dl712* (blue trace). The percentage of mock-infected cells that were stained for TNFR1 was 93%, as compared to 16% and 18%, respectively, for *rec700* and *dl712*. In this same experiment, cell surface Fas was also internalized by *rec700* and *dl712* (Fig. 18B). Thus, Ad infection removes TNFR1 from the cell surface, as is the case with Fas.

The mutant used in the above experiment, *dl712*, overexpresses RID and E3-14.7K, and expresses very little of the other E3 proteins. To determine whether RID and/or E3-14.7K is involved in internalization of TNFR1 in Ad-infected HeLa cells, the same experiment was performed using *dl712* and additional E3 mutants: *dl309*, which lacks RID α , RID β , and E3-14.7K; *dl753*, which lacks RID α but expresses RID β and E3-14.7K; and *dl764*, which lacks RID β but expresses RID α and E3-14.7K. The deletions in these mutants do not affect expression of any other Ad proteins. The results are shown in Figure 19.

With *rec700* and *dl712*, TNFR1 was removed from the cell surface such that only 29% and 24%, respectively, of cells were stained for TNFR1 as compared to 92% with mock-infected cells (Fig. 19A). With *dl309*, *dl753*, and *dl764* infected cells, 84%, 85%, and 84%, respectively, were stained for TNFR1, indicating that these mutants did not induce removal of TNFR1 from the cell surface. Cell surface Fas was also examined in this same experiment.

rec700 and *dl712* cleared Fas whereas *dl309*, *dl753*, and *dl764* did not (Fig. 19B). Thus, RID is required to remove TNFR1 from the surface of Ad-infected cells, as is the case with Fas.

As a means to determine whether RID is sufficient to remove TNFR1 from the cell surface, HeLa cells were infected with the Ad vector named 231-10. This vector will be described in detail in Example 10 below. In brief, 231-10 lacks the E1A, E1B, and E3 transcription units. The deleted E1A plus E1B regions are replaced with an expression cassette wherein all the E3 proteins are expressed from the human cytomegalovirus (CMV) promoter. Because 231-10 lacks E1A, viral genes in the vector backbone are not expressed; only the E3 proteins are expressed from the CMV promoter. Thus, the vector serves as an essentially inert vehicle by which E3 genes can be delivered into cells and the properties of their proteins studied.

HeLa cells were mock-infected or infected with the 231-10 vector, and cell surface TNFR1 was examined by flow cytometry at 24 h and 48 h p.i. as described above. At 24 h p.i., the percentage of cells bearing TNFR1 was reduced from 93% to 35%, and by 48 h the percentage was reduced to 11% (Fig. 20). This time course of TNFR1 down-regulation correlates with expression of the E3 proteins. In a parallel experiment, Fas was nearly completely cleared by 24, 36, and 48 h p.i. (data not shown). Thus, TNFR1 and Fas are removed from the cell surface by the E3 proteins expressed by 231-10. RID is undoubtedly the E3 protein responsible for the removal of these death receptors.

The ability of Ad and the RID protein to remove TNFR1 from the cell surface was examined using the biotin-streptavidin system (Stewart et al., 1995) to detect TNFR1. Multiple dishes of A549 cells were mock-infected or infected with 50 PFU/cell of *rec700* (wild-type). At 16 h p.i., cell surface proteins in mock- and Ad-infected cells were labeled using biotin. Ad-infected cells in other dishes were also labeled with biotin at 18, 20, 22, 24, and 30 h p.i. Proteins were extracted using buffer containing 0.5% NP-40, and were incubated with protein A-Sepharose CL-4B attached to the B/O:2/18/91 rabbit antiserum against TNFR1. After washing, proteins were solubilized, subjected to SDS-PAGE, and transferred to membranes. Membranes were incubated with peroxidase-conjugated streptavidin (Sigma), and proteins were visualized using ECL (Amersham).

In this assay, if Ad infection has resulted in the removal of TNFR1 from the cell surface, then TNFR1 will not be available for biotinylation and therefore TNFR1 will not be detected. As shown in Fig. 21, similar amounts of TNFR1 were obtained from mock- or *rec700*-infected cells at 16 h p.i. With *rec700*, TNFR1 declined from 18 to 30 h p.i. until only small amounts were detected. Thus, as was the case when TNFR1 was detected by flow cytometry, Ad infection results in markedly decreased amounts of cell surface TNFR1.

The ability of the 231-10 Ad vector to down-regulate cell surface TNFR1 as determined with the biotin-streptavidin assay was also examined. As discussed above, 231-10 expresses only Ad E3 proteins. Cells were mock-infected, infected with 50 PFU/cell of *rec700* (wild-type), or infected with 250 PFU/cell of 231-10. At different days p.i., cells were biotinylated and TNFR1 detected as described above. As expected, most of the TNFR1 was cleared by *rec700* at 1 day p.i. (Fig. 22A, compare lanes a and b). With 231-10, reduced amounts of TNFR1 were detected by 1 day p.i., and by 5 days p.i. the TNFR1 levels declined to those of *rec700*. The levels of TNFR1 in mock-infected cells were similar after 5 days to those after 1 day (Fig. 22A, compare lane h with lane a). Therefore, the reduction at 5 days seen with 231-10 is not due to a non-viral event associated with maintaining the cells in dishes for 5 days. These results indicate that the E3 proteins expressed by the 231-10 vector, presumably RID, are sufficient to clear TNFR1 from the cell surface.

The accumulation of RID β in these same cell extracts was also examined by standard immunoblot using the rabbit P118-132 antiserum (Stewart et al., 1995). With *rec700*, RID β was abundant after 1 day (Fig. 22B, lane b). The multiple bands on RID β are species of RID β that are differentially O-glycosylated and phosphorylated. With 231-10, RID β was detected after 2 days, and it increased dramatically in abundance from days 3-5 (Fig. 22B, lanes c-g). Therefore, as expected, the accumulation of RID β in this experiment correlated inversely with the decline in cell-surface TNFR1.

These results obtained using the B/O:2/18/91 antibody in the biotin-streptavidin and flow cytometry assays to detect TNFR1 are consistent. Thus, it is believed that RID is necessary to efficiently down-regulate cell surface TNFR1 in Ad-infected cells. The results with 231-10 indicate that RID is sufficient to down-regulate TNFR1, with the caveat that the E3 14.7K and gp19K proteins, and possibly the E3 12.5K and 6.7K proteins, are expressed by 231-10.

To determine if RID is responsible for clearance of cell-surface TNFR1, the following Ad E3 mutants were used: *d1748*, which overexpresses RID β but lacks RID α ; and *d1798*, which overexpresses RID α but lacks RID β . A549 cells were mock-infected or infected with 50 PFU/cell of *rec700*, *d1748*, or *d1798*, or infected with 25 PFU/cell each of *d1748* and *d1798*. At 26 h p.i. cells were biotinylated and TNFR1 examined as described above. As a positive control, a dish of mock-infected cells was treated with TNF, and the cell extract was examined for TNFR1. As expected, TNF removed most of the TNFR1 from the cell surface (Fig. 23A, lanes a and b).

The results with the viruses are shown in Fig. 23A, lanes c-f. With *rec700* (wild-type)-infected cells, only small amounts of TNFR1 were detected (lane c). With *d1748*

(RID α ⁻, RID β ⁻) and *dl798* (RID α ⁺, RID β ⁻), high to intermediate levels of TNFR1 were observed, indicating that RID α and RID β are required for efficient clearance of TNFR1. When cells were co-infected with *dl748* and *dl798*, TNFR1 was reduced to levels comparable to *rec700*-infected cells (lanes f and c). This result indicates that the mutants complement
5 (*dl748* provides RID β , *dl798* provides RID α), and that both RID α and RID β are required for efficient removal of TNFR1 from the cell surface. Figure 23B shows a standard immunoblot for E1B-19K from the same extracts that were analyzed for biotinylated TNFR1. Similar amounts of E1B-19K were detected with all viruses. Therefore, differences in TNFR1 levels seen with these viruses are not due to differences in infection efficiency by the viruses.

10 The partial clearance of TNFR1 observed with these RID α ⁻ and RID β ⁻ mutants is consistent with the flow cytometry data in Fig. 19. These results suggest that there may be a mechanism in addition to RID that down-regulates cell-surface TNFR1 in Ad-infected cells. However, clearly, most of the down-regulation of TNFR1 requires RID.

In summary, RID is required to remove TNFR1 from the surface of Ad-infected cells.
15 RID is also sufficient for removal of TNFR1 as indicated by the experiment with the 231-10 vector, with the caveat that the 231-10 vector also expresses other E3 proteins. RID expressed by the 231-10 vector is also sufficient to remove Fas from the cell surface, again, with the same caveat. However, the down-regulation of TNFR1 and Fas by 231-10 is almost certainly due to RID, because the mutant mapping data with E3 mutants have provided no
20 indication that other E3 proteins play any role in down-regulating these death receptors.

Example 9

This example demonstrates that the 231-10 vector prevents rejection of human cancer cells transplanted into immunocompetent mice.

25 Cells or tissues transplanted into immunocompetent recipients are usually destroyed (rejected) by immune killer cells of the recipient. Rejection begins within 1-2 days, and therefore is mediated by the innate immune system including macrophages and NK cells. Specific CTL formed after about 5-7 days also play a major role in transplant rejection. As discussed above in Example 7, RID inhibits NK- and CTL-killing of Ad-infected cells and
30 thus should also be able to inhibit NK- and CTL-mediated rejection of transplanted cells or tissues.

This idea was tested by determining whether the E3 proteins expressed by the 231-10 vector will permit human cancer A549 cells to grow as a tumor in immunocompetent C57BL/6 (H-2^b) mice. Human cancer cells normally will be rejected when transplanted in

C57BL/6 mice. However, RID should inhibit rejection by removing Fas and TNFR1 from the transplanted cells. E3-14.7K may also prevent rejection.

5 A549 cells mock-infected or infected with 50 PFU/cell of 231-10. After 48 h, 2×10^6 cells (in 100 μ l) were injected subcutaneously into each hind limb flank of female C57BL/6 mice. At 18 days post-injection, the mice were sacrificed and the site of injection was examined following removal of the skin. With mice that received mock-infected cells, there was a pin-point mass on one flank, and no mass at all on the other flank (data not shown). With the 231-10-infected cells, there were significant tumor masses on both flanks (Fig. 24). The tumors were opaque and ellipsoid in shape. The left-flank tumor was attached to the muscle. The right-flank tumor, which is shown in higher magnification in Fig. 25, was attached to both the muscle and skin. The size of the tumor obtained with 231-10-infected cells was many times larger than what would be observed from the initial bolus of cells injected (2×10^6 cells are barely visible to the naked eye). Thus, the cells grew into a tumor.

10 In the second experiment, mock-infected and 231-10-infected A549 cells (at 2 days p.i. in culture, 50 PFU/cell) were used, both live cells as well as cells that were killed by freezing and thawing. These cells were injected into each hind limb of C57BL/6 and Balb/c mice, 2×10^7 cells per injection. As is the case with C57BL/6, the Balb/c mice are fully immunocompetent. There were four mice of each strain. Mouse 1 received killed uninfected A549 cells, mouse 2 received live A549 cells, mouse 3 received killed 231-10-infected cells, and mouse 4 received live 231-10-infected cells. Mice were harvested at 15 days following injection. No tumors were observed in either mouse strain with killed cells. With the C57BL/6 mouse that received uninfected live cells, there was no growth on one flank and a very small mass on the other flank. With the Balb/c mouse that received live uninfected cells, there were small flat masses on each flank. However, with both the C57BL/6 and the Balb/c mouse that received 231-10-infected cells, there were much larger ellipsoid masses (tumors) on both hind flanks. These tumors resembled the tumors shown in Figs. 24 and 25. Therefore, as was the case in the first experiment, the 231-10 vector allowed A549 cells to form tumors in immunocompetent mice.

25 One of the 231-infected cell tumors from the C57BL/6 mouse was examined for expression of the E3 proteins known to be synthesized in cultured cells by 231-10. Proteins were extracted from the tumor, and the RID β , 14.7K, and gp19K proteins assayed by immunoblot. As shown in Fig. 26, all three proteins were detected. This result provides very strong evidence that the cells originally infected with 231-10, at the very minimum, persisted in the mouse. It is very likely that these cells grew as well, considering that tumors were formed. It is not likely that the 231-10 vector replicated in these cells, because the vector

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lacks the E1A gene. Most likely, as the A549 cells proliferated in the mouse, a portion of the input vector was segregated into the daughter cells.

In summary, the E3 proteins expressed from the 231-10 vector have permitted the growth of human A549 cancer cells to form tumors in C57BL/6 and Balb/c mice. The tumors would not have been able to form unless they were protected from destruction by the immune system. These results argue strongly that the E3 proteins should prevent immune rejection of other types of transplanted cells and tissues. Thus, the 231-10 vector has the potential to be used in tissue or cell transplants to prevent rejection of the tissues or cells.

Example 10

This example illustrates the construction and properties of the 231-10 vector.

Features of 231-10

The 231-10 vector is a human adenovirus serotype 5 (Ad5) vector. It can be viewed as a "transient transfection" system, analogous to that obtained when a plasmid expression vector is transfected into cells. The basic features of the 231-10 vector are outlined in the schematic shown in Fig. 27 and the entire DNA sequence of the genome of 231-10 is given in Fig. 28.

The horizontal bar in Fig. 27 depicts the linear double-stranded DNA genome. The base pairs (nucleotides) are numbered from 1 to 34427 (see Fig. 28), from left to right in Fig. 27. Nucleotides 342-3523 are deleted, removing all the genes in the Ad E1A and E1B transcription units (collectively known as E1). Nucleotides 28133-30818 are also deleted, removing all the genes in the E3 transcription unit. In place of E1, an expression cassette has been inserted, in which the E3 genes are expressed from the human cytomegalovirus immediate early promoter-enhancer (CMV). This E3 expression cassette contains the E3 genes from the virus named *pm734.1*, which is a derivative of the virus named *rec700* (Tollefson et al., *Virol.* 220:152-162, 1996). *rec700* is an Ad5-Ad2-Ad recombinant that has the Ad2 version of the E3 genes for the 12.5K, 6.7K, gp19K, and RID α proteins, and the Ad5 version of the E3 genes for the RID β and 14.7K proteins. The E3 cassette in 231-10 contains all the E3 genes from *pm734.1*. Notably, there are two missense mutations in the *adp* gene (which encodes the Adenovirus Death Protein [ADP], previously named E3-11.6K) (Tollefson et al., *supra*). These two mutations eliminate the first two methionine codons in the *adp* gene, thereby precluding synthesis of functional ADP (Tollefson et al., *supra*).

The 231-10 vector was designed to have the following properties. First, since the E1A genes are lacking, the vector should not replicate (efficiently) on most cell lines. Therefore, Ad early and late proteins will not be expressed and Ad DNA will not replicate. (It is known that Ad mutants lacking E1A do replicate their DNA and express late proteins at low levels when high multiplicities of infection are used and the infection is allowed to

proceed for several days. This is also true for 231-10 [not shown].) Second, the E3 proteins should be expressed in an E1A-independent manner from the CMV promoter/enhancer.

Thus, 231-10 is an essentially inert vehicle that can deliver the Ad E3 proteins into cells without having other Ad proteins expressed, at least for the first approximately 3 days

5 following infection. Even after 3 days, other Ad proteins should be expressed only in very small amounts, much less than the E3 proteins.

Construction of Ad 231-10

(a) The genes of the E3 transcription unit were excised from *pm734.1* (*pm734.1* is *rec700* with mutations of the Met1 and Met41 codons in the *adp* gene. *rec700* is the same as
10 Ad5 but with the Ad2 EcoRI-D fragment substituted for the corresponding Ad5 EcoRI-C fragment). The *pm734.1* SrfI-NdeI-D fragment (3560 bp) was blunt-end using the Klenow enzyme and cloned into the SmaI site of the pBluescriptSK(+) vector (Stratagene), resulting in plasmid p1721 which has the whole E3 transcription unit of *pm734.1* (-39 to 3521) flanked
15 by SalI-BstXI-SacII-NotI-XbaI-SpeI-BamHI sites situated upstream from the E3 sequences and PstI-EcoRI-EcoRV-HindIII-ClaI-SalI-XhoI sites situated downstream from the E3 sequences.

(b) The BamHI-SalI-A fragment (3605 bp) of p1721 was subcloned between the BamHI-XhoI sites of plasmid pCDNA3.1zeo(+) (Invitrogen), resulting in plasmid p181 in which E3 genes are under control of the CMV promoter-enhancer.

20 (c) The MfeI-ClaI fragment of p181 (4328 bp), corresponding to the CMV promoter-E3 genes from the *pm734.1* expression cassette, was subcloned between the EcoRI-ClaI sites of plasmid pΔE1splA (Microbix Biosystems Inc., Toronto), resulting in plasmid p231 which has the CMV-E3 expression cassette flanked by Ad5 genomic sequences (Ad5 map units 0-1 and 9.8-16.1). The orientation of the CMV-E3 expression cassette is right-to-left (opposite to
25 the Ad E1 and major late transcription units).

(d) Plasmid p231 was cotransfected along with plasmid pBHG10 (Microbix Biosystems Inc., Toronto) into 293 cells resulting in plaques of recombinant virus 231-10. The virus has deletions of E1 (Ad5 nt 342-3523) and E3 (Ad5 nt 28133-30818), and has the CMV-E3 expression cassette in place of the E1 deletion.

30 The 231-10 Vector Expresses the E3 RID, 14.7K, and gp19K proteins.

The E3 proteins are expected to be synthesized from the E3 expression cassette in 231-10. To demonstrate that this is so, separate dishes of A549 cells were infected with 250 PFU/cell of 231-10, then at 0-5 days p.i. protein extracts were examined for the E3 RID, 14.7K, and gp19K proteins using standard immunoblot procedures (Tollefson et al., Nature
35 392:726-730 (1998)). In one dish, 231-10-infected cells were treated with 1-β-D-

arabinofuransylcytosine (araC) at 2 h p.i., then proteins were extracted at 1 day p.i. RID β , 14.7K, and gp19K were readily detected at 2 days p.i., and their abundance increased until the end of the experiment at 5 days p.i. (Fig. 29, lanes d-g). On longer exposures of the gel shown in Fig. 29, a trace of RID β , 14.7K, and gp19K can be seen at 1 day p.i. (not shown).

5 In the experiment shown in Fig. 29, one dish of cells was treated with araC. AraC inhibits Ad DNA replication, and therefore Ad late genes cannot be expressed. As shown in Fig. 29, small amounts of RID β and gp19K were detected in the araC-treated cells; 14.7K was also detected in longer exposures of the gel (lane A). Therefore, as expected, E3 proteins are synthesized by 231-10 without replication of the vector Ad DNA.

10 These results demonstrate that the RID β , 14.7K, and gp19K proteins are expressed in 231-10-infected cells. In another experiment, the levels of RID β at 4 or 5 days p.i. were roughly similar to those of *rec700*-infected cells at 1 day p.i. (see Fig. 22). Bearing in mind that *rec700* has replicated by 1 day p.i. and therefore has expressed higher levels of RID β from more templates, the quantities of RID β , 14.7K, and gp19K observed with 231-10, which
15 does not replicate (or only replicates in small amounts at 4 or 5 days p.i.), are quite high. The synthesis of the E3 12.5K and 6.7K proteins by 231-10 has not been examined. Although not shown directly in Fig. 29, the RID α polypeptide is also expressed by 231-10. This can be deduced from the observation that 231-10 exhibits the expected functions of RID, namely it clears Fas and TNFR1 from the surface of infected cells (see Example 8.). These functions
20 require both RID α and RID β .

Indirect immunofluorescence was also used to study the expression of the gp19K, RID β , and 14.7K proteins in A549 cells infected with 231-10. At 2 days p.i., the gp19K and RID β proteins were visualized as described previously (Tollefson et al., *Nature* 392:726-730 (1998); Hermiston et al., *J. Virol.* 67:5289-5298 (1993)) and the 14.7K protein was stained
25 using a rabbit antiserum directed against a TrpE-14.7K fusion protein (Tollefson and Wold, *J. Virol.* 62:33-39 (1988)). Strong staining of gp19K was observed in a pattern typical of the endoplasmic reticulum (Fig. 30A), as has been observed with *rec700* (Hermiston et al., supra). The pattern for RID β was also similar to that seen with *rec700*, i.e. staining of the Golgi, other membranes, and the plasma membrane (Fig. 30B; Tollefson et al., *Nature*
30 392:726-730 (1998)). The 14.7K protein staining was diffuse in the cytoplasm (Fig. 30C), which again is typical of *rec700* (unpublished results). These results establish that the E3 gp19K, RID, and 14.7K proteins localize to the same or similar intracellular compartments as they do in wild-type Ad-infected cells.

In view of the above, it will be seen that the several advantages of the invention are
35 achieved and other advantageous results attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

- 5 All references cited in this specification are hereby incorporated by reference. The discussion of references herein is intended merely to summarize the assertions made by their authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinency of the cited references.

What is Claimed is:

1. A method for inhibiting apoptosis of a cell comprising treating the cell with an effective amount of a Receptor Internalization and Degradation (RID) complex.
2. The method of claim 1 wherein the treating step comprises administering to the cell a polynucleotide encoding the RID complex and wherein the RID complex is expressed in the cell.
3. The method of claim 2 wherein the polynucleotide comprises a recombinant adenovirus vector.
4. The method of claim 3 wherein the recombinant adenovirus vector is 231-10.
5. The method of claim 3 wherein the cell expresses Fas, TNFR-1, DR3, TRAIL-R1, or TRAIL-R2.
6. The method of claim 5 wherein the cell is a leukocyte.
7. The method of claim 5 wherein the cell comprises a transplant tissue.
8. The method of claim 1 wherein the treating step comprises administering the RID complex to the cell.
9. The method of claim 8 wherein the RID complex is administered with a carrier which facilitates delivery of the RID complex into the cell.
10. A method for decreasing apoptosis of target cells in a patient comprising treating the patient with an effective amount of a Receptor Internalization and Degradation (RID) complex.
11. The method of claim 10 wherein the treating step comprises administering to the patient a polynucleotide encoding the RID complex and wherein the polynucleotide is internalized in the target cells and the RID complex is expressed.
12. The method of claim 11 wherein the polynucleotide comprises a recombinant adenovirus vector.
13. The method of claim 12 wherein the recombinant adenovirus vector is 231-10.
14. The method of claim 10 wherein the patient suffers from a degenerative disease or an immunodeficiency disease.
15. The method of claim 10 wherein the treating step comprises administering the RID complex to the patient.
16. The method of claim 15 wherein the RID complex is administered with a carrier which facilitates delivery of the RID complex into the cells.
17. A method for decreasing leukocyte apoptosis in a patient comprising:
 - (1) withdrawing leukocytes from the patient,
 - (2) treating the leukocytes with an effective amount of a RID complex, and
 - (3) administering the treated leukocytes to the patient.

18. The method of claim 17 wherein the treating step comprises administering to the leukocytes a polynucleotide encoding the RID complex wherein the RID complex is expressed in the leukocytes.

19. The method of claim 18 wherein the polynucleotide comprises a recombinant adenovirus vector.

20. The method of claim 19 wherein the recombinant adenovirus vector is 231-10.

21. The method of claim 17 wherein the treating step comprises administering the RID complex to the leukocytes.

22. The method of claim 21 wherein the RID complex is administered with a carrier which facilitates delivery of the RID complex into the leukocytes.

23. A composition comprising a Receptor Internalization and Degradation (RID) complex and a carrier suitable for facilitating delivery of the RID complex into a cell.

24. A recombinant adenovirus comprising a polynucleotide encoding a Receptor Internalization and Degradation (RID) complex operably linked to a promoter, wherein the adenovirus is replication defective and wherein the polynucleotide is expressed upon infection of a eukaryotic cell with the adenovirus.

25. The recombinant adenovirus vector of claim 24 consisting of 231-10.

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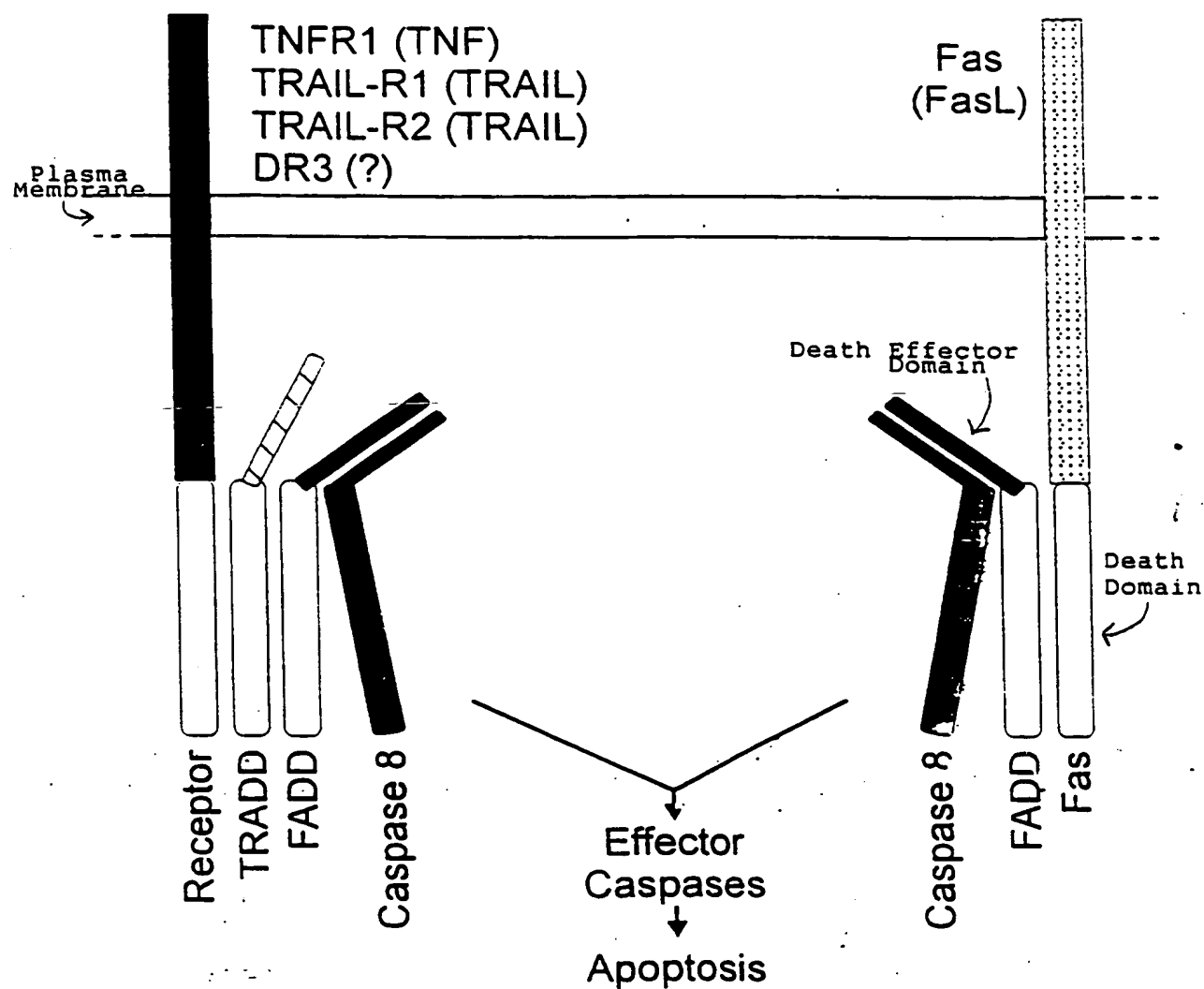


FIGURE 2

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RID COMPLEX

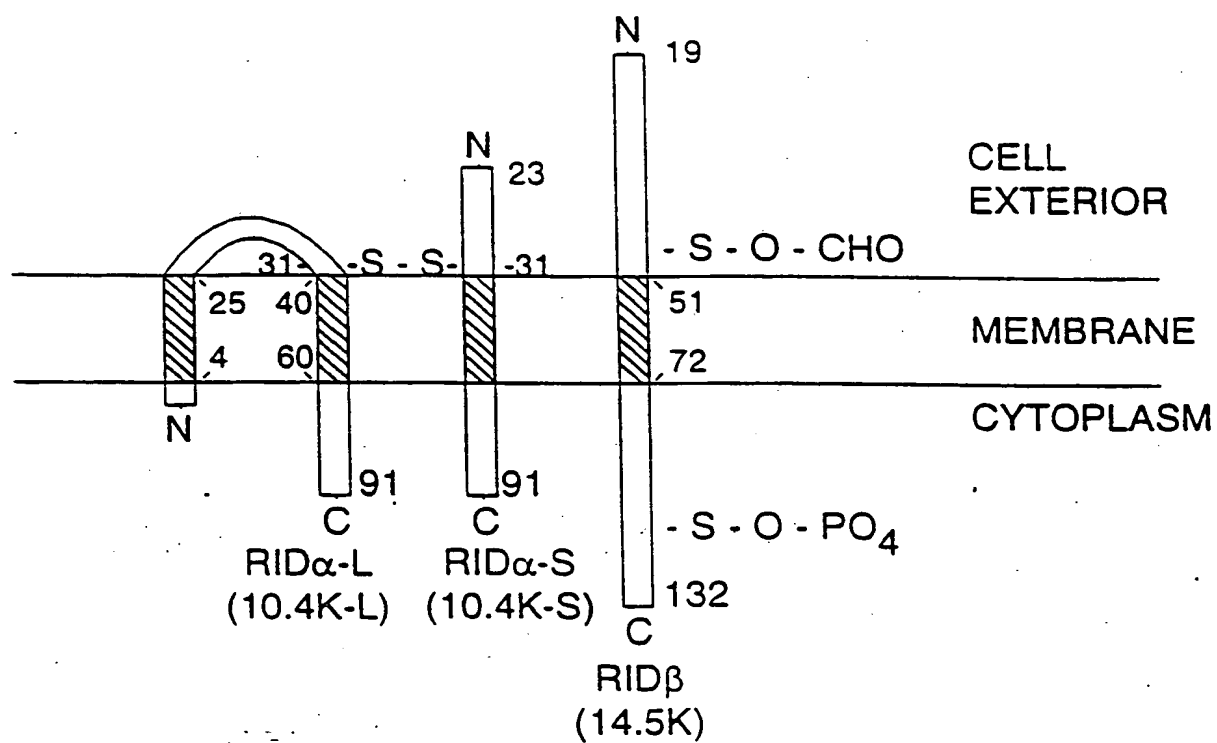


Figure 3

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RID α -L (10.4K-L)

10 20
M I P R V L I L L T L V A L F C A C S T L A A V A H I E
signal sequence

30 40 50
V D C I P P F T V Y L L Y G F V T L I L I C S L V T V V
* transmembrane

60 70 80
I A F I Q F I D W V C V R I A Y L R H H P Q Y R D R T I

90
A D L L R I L

Figure 4A

RID α -S (10.4K-S)

10 20
A V A H I E V D C I P P F T V Y L L Y G F V T L I L I C
* transmembrane

30 40 50
S L V T V V I A F I Q F I D W V C V R I A Y L R H H P Q

60
Y R D R T I A D L L R I L

Figure 4B

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Pre-RID β (14.5K)

10 20
M K F T V T F L L I I C T L S A F C S P T S K P Q R H I
 signal sequence

30 40 50
S C R F T R I W N I P S C Y N E K S D L S E A W L Y A I

60 70 80
I S V M V F C S T I L A L A I Y P Y L D I G W N A I D A
 Transmembrane

90 100 110
 M N H P T F P A P A M L P L Q Q V V A G G F V P A N Q P

120 130
 R P P S P T P T E I S Y F N L T G G D D
 * *

Figure 4CMature-RID β (14.5K)

10 20
 S P T S K P Q R H I S C R F T R I W N I P S C Y N E K S

30 40 50
 D L S E A W L Y A I I S V M V F C S T I L A L A I Y P Y
 Transmembrane

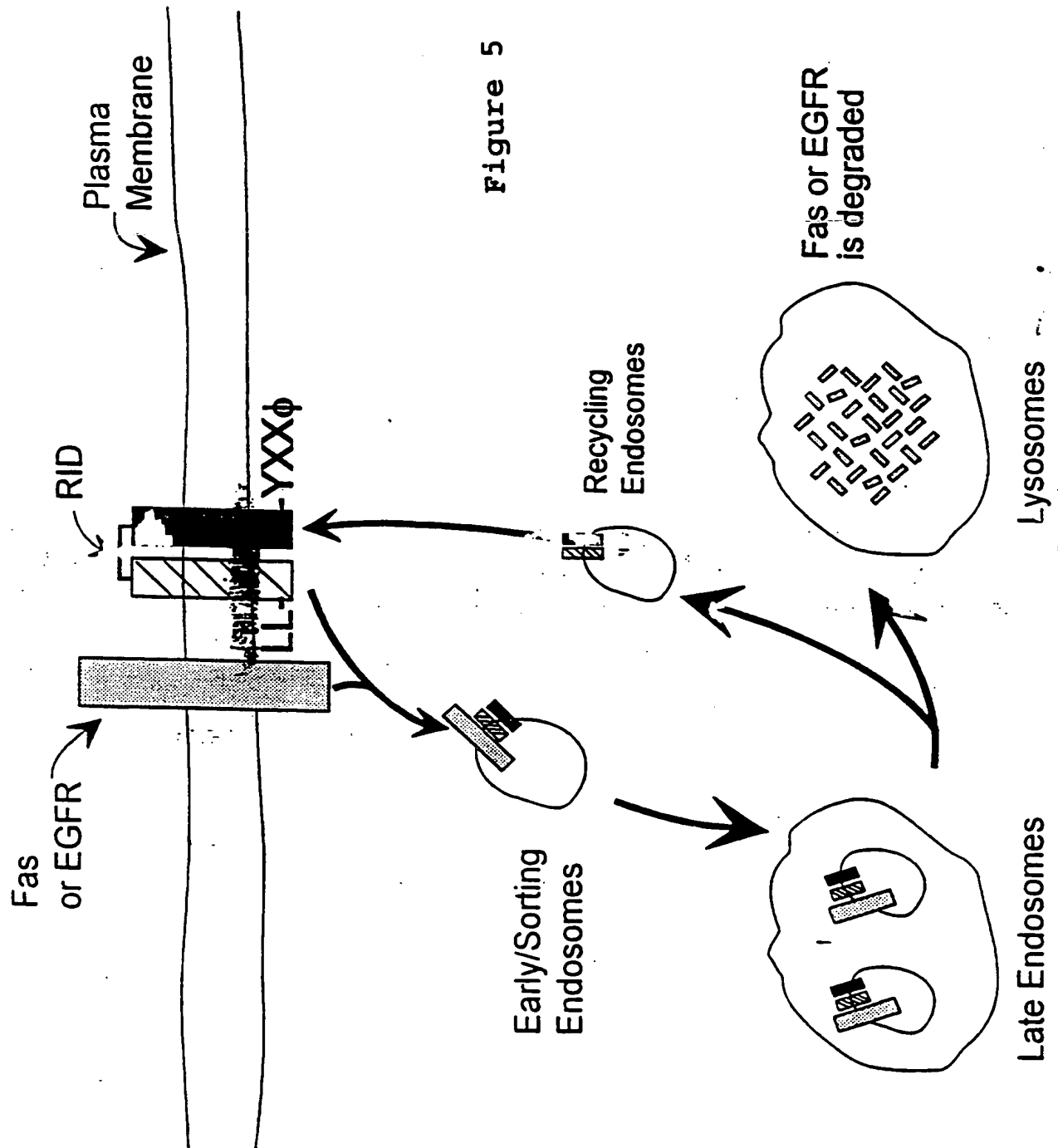
60 70 80
 L D I G W N A I D A M N H P T F P A P A M L P L Q Q V V

90 100 110
 A G G F V P A N Q P R P P S P T P T E I S Y F N L T G G
 * *

D D

Figure 4D

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rec700, anti-DPB



Figure 6A

rec700, DAPI

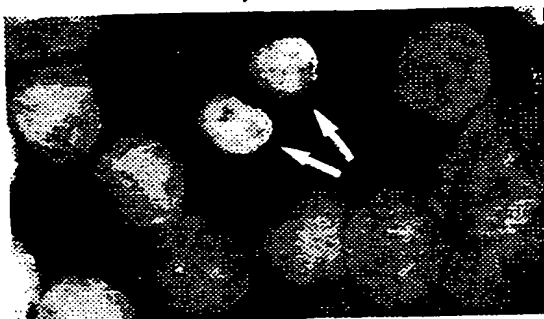


Figure 6B

RID, anti-RID β

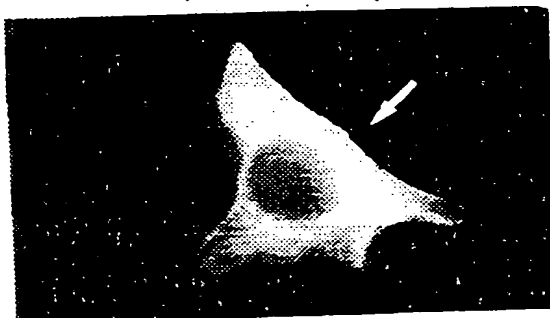


Figure 6C

RID, DAPI

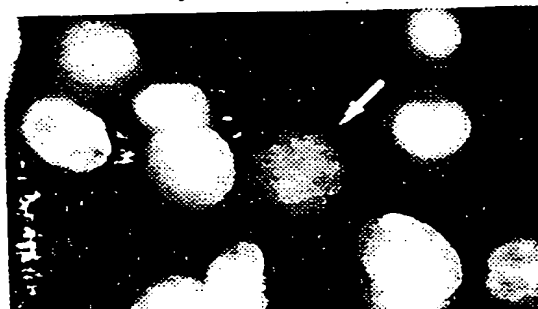


Figure 6D

Figure 7A

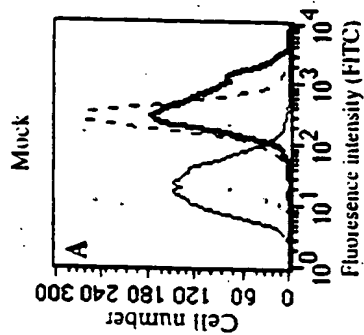


Figure 7B

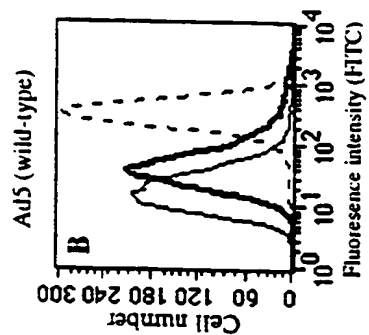


Figure 7C

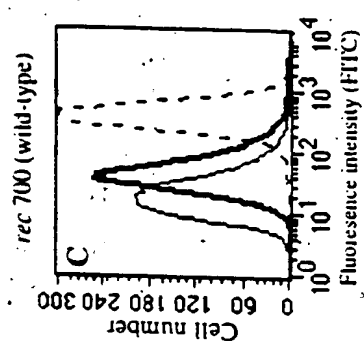
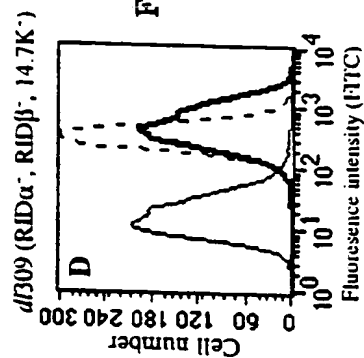


Figure 7D



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Figure 7F

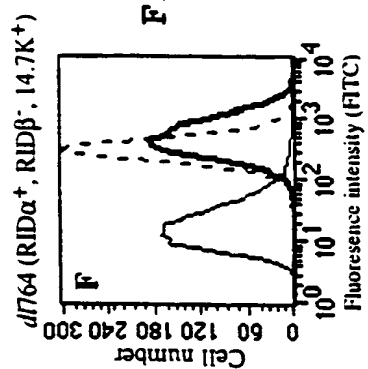


Figure 7H

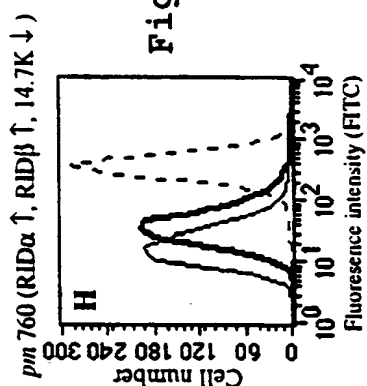


Figure 7E

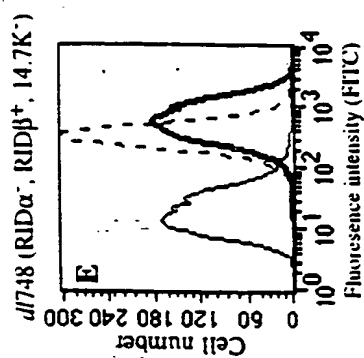
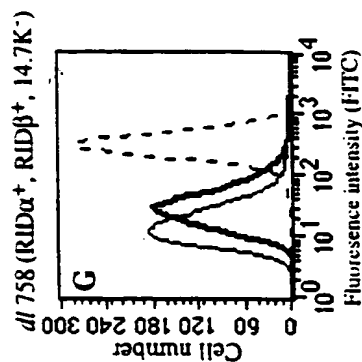


Figure 7G



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Figure 8B

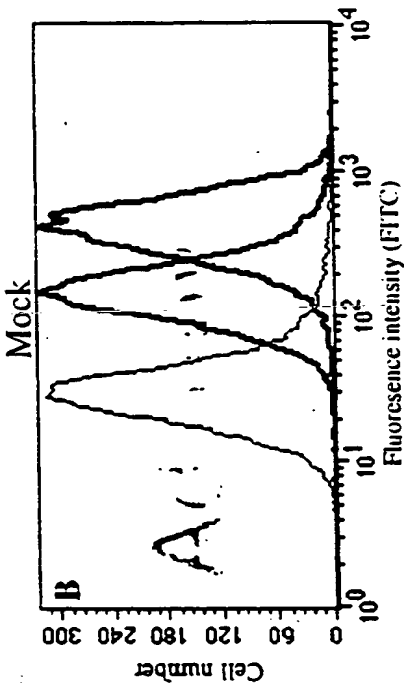


Figure 8A

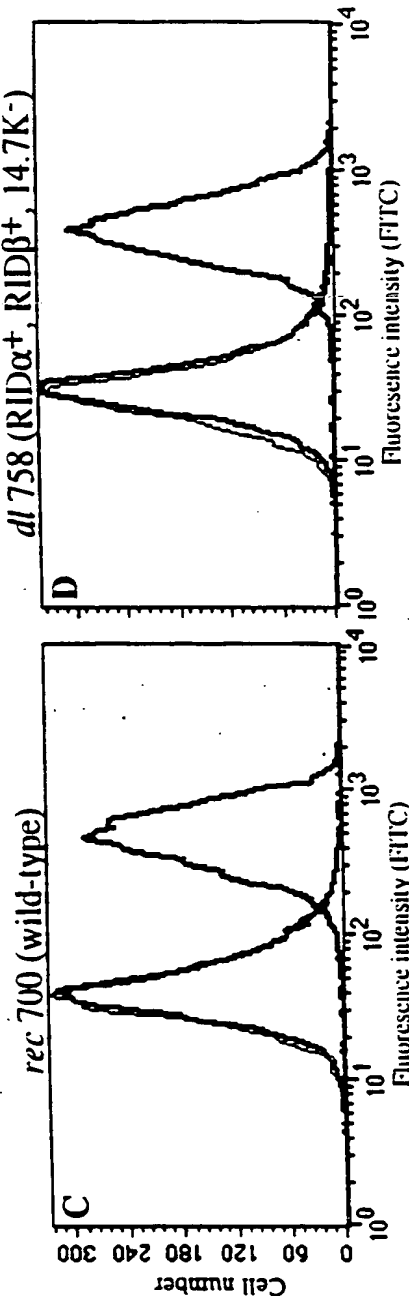
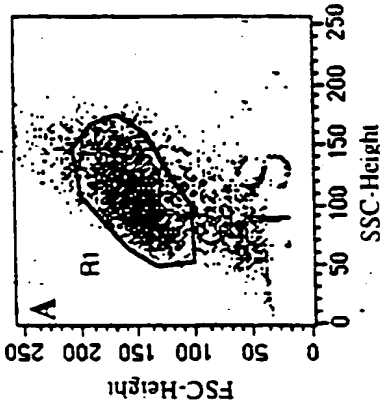


Figure 8D

Figure 8C

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Figure 8F

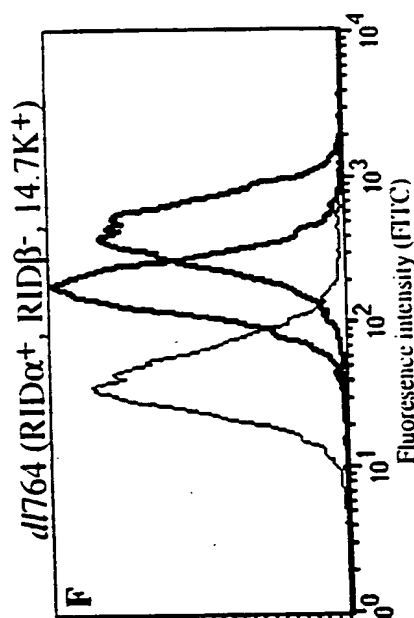


Figure 8E

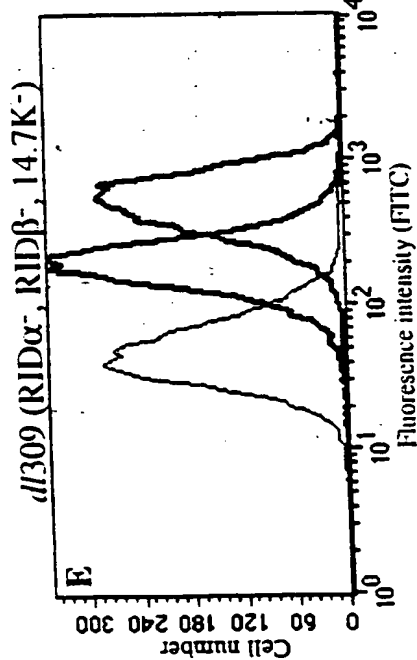


Figure 8H

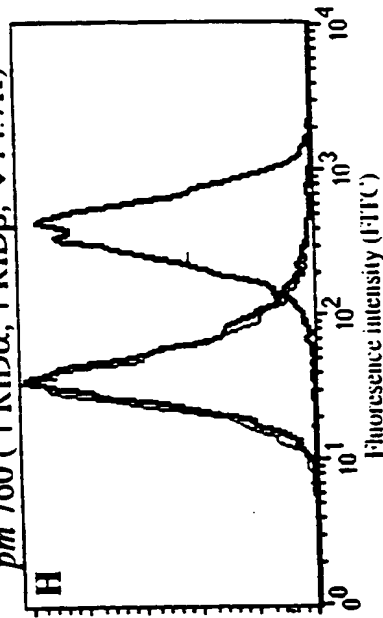


Figure 8H

Figure 8G

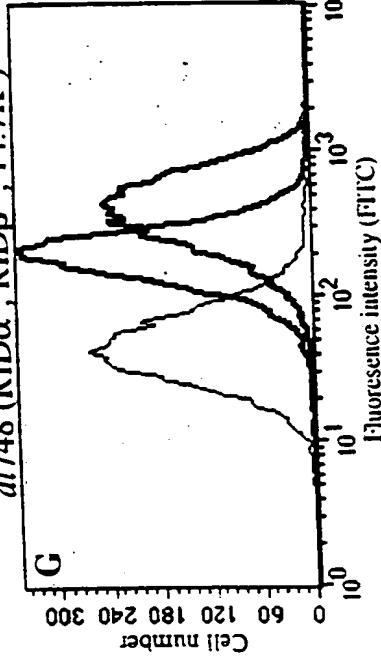


Figure 8G

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Figure 9A

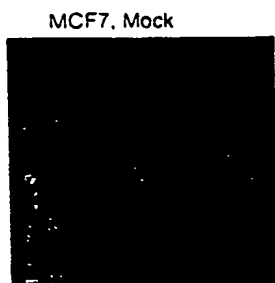


Figure 9B

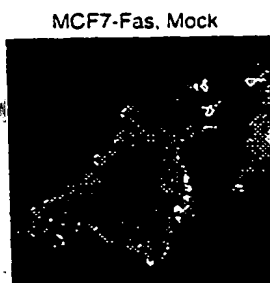


Figure 9C

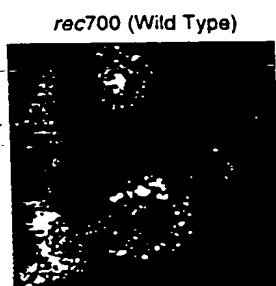


Figure 9D

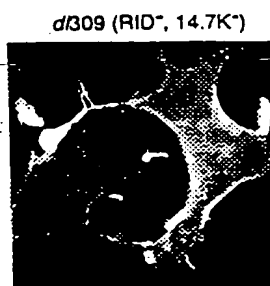


Figure 9E

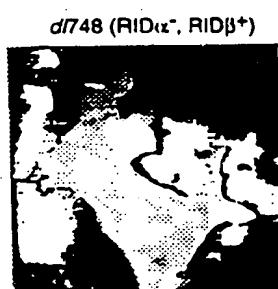


Figure 9F

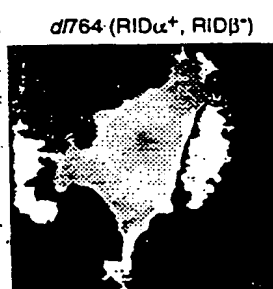


Figure 9G

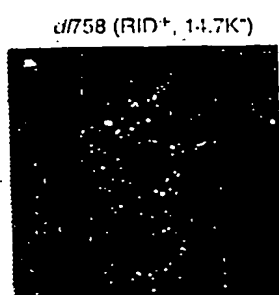
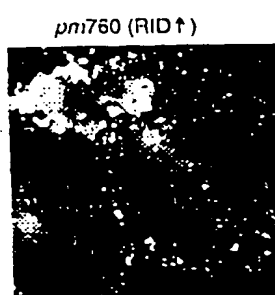


Figure 9H



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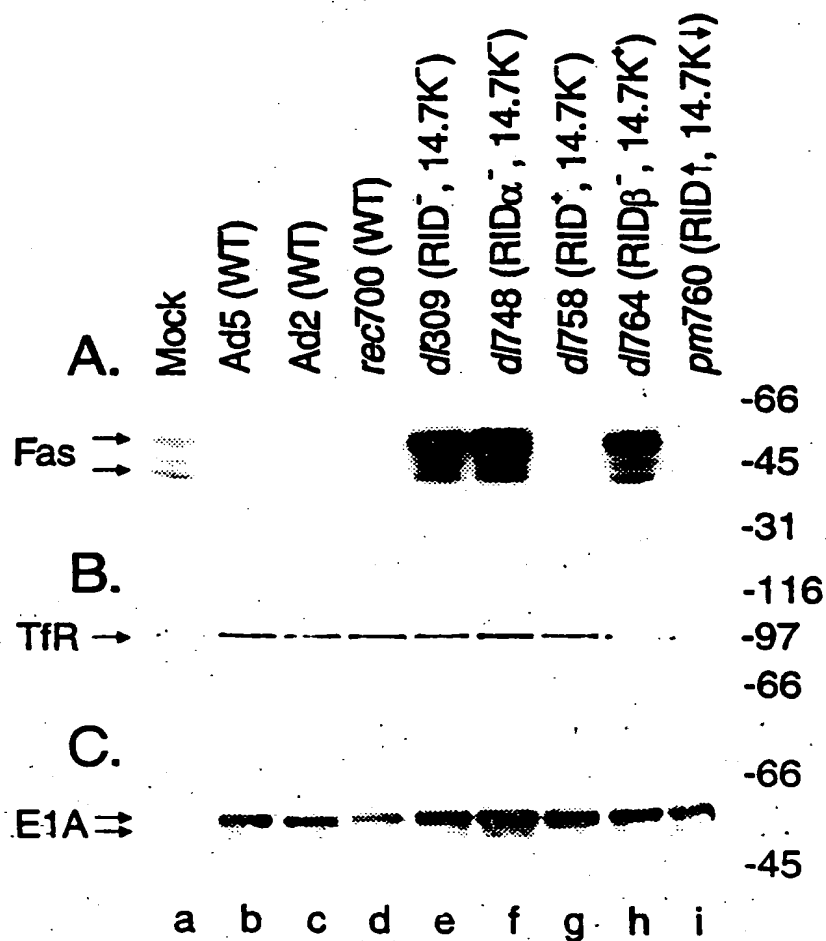


Figure 10

Figure 11B

RID α , anti-Fas



RIDβ, anti-Fas

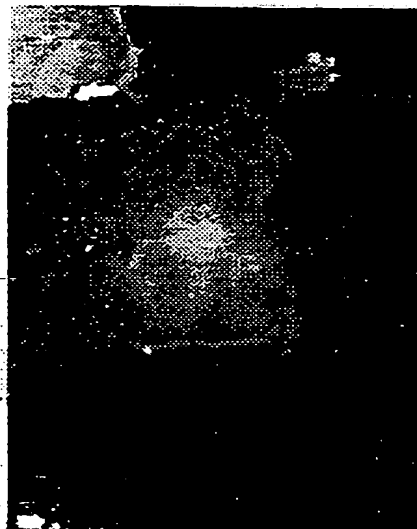
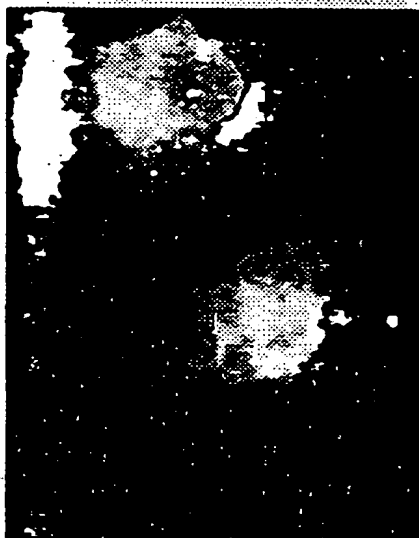


Figure 11D

RIDα, anti-RIDα



RIDβ, anti-RIDβ

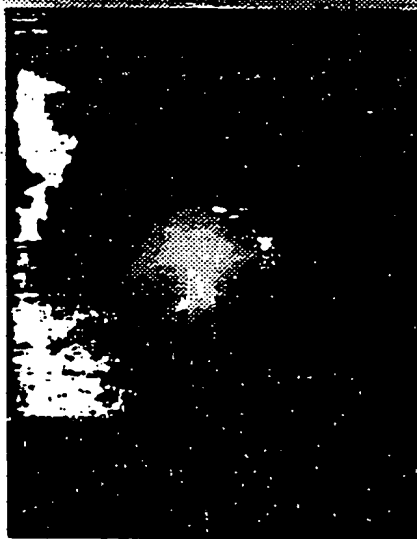


Figure 11A

Figure 11C

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Figure 11F

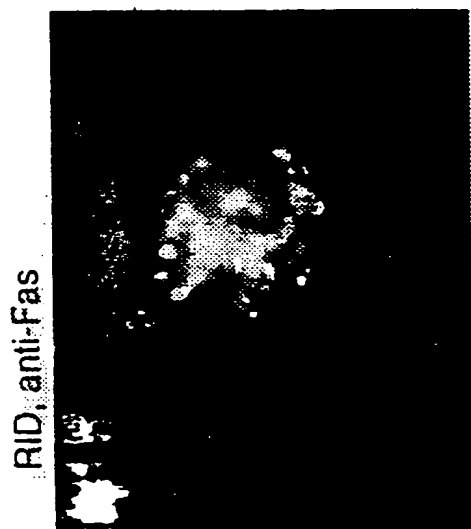


Figure 11H

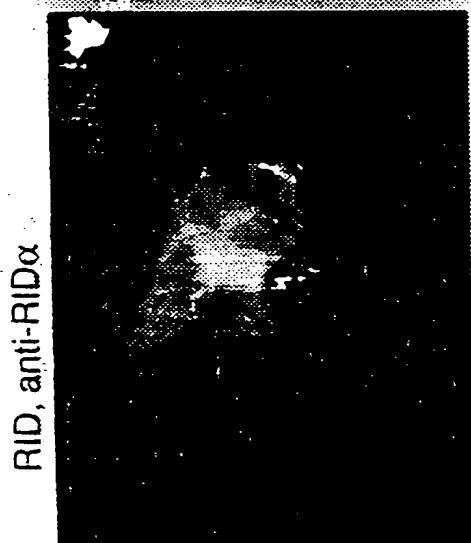
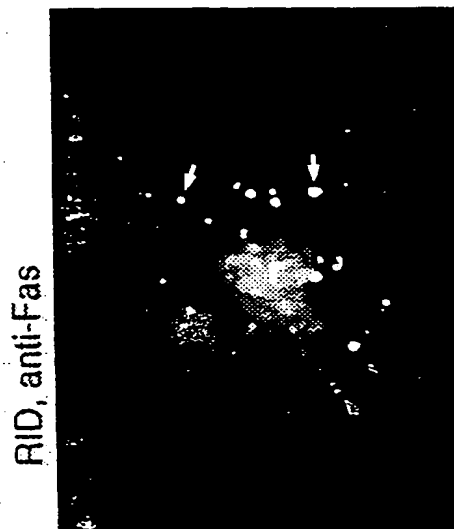


Figure 11E

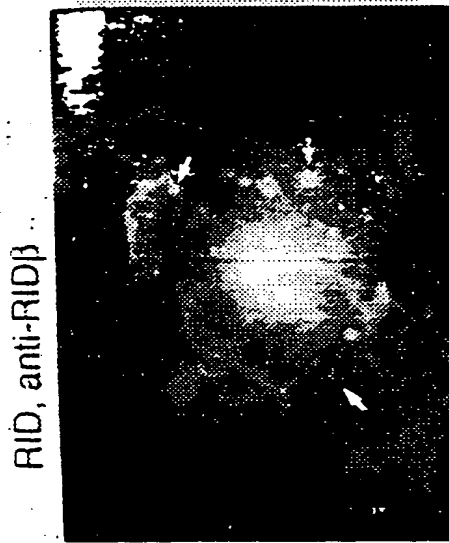


Figure 11G

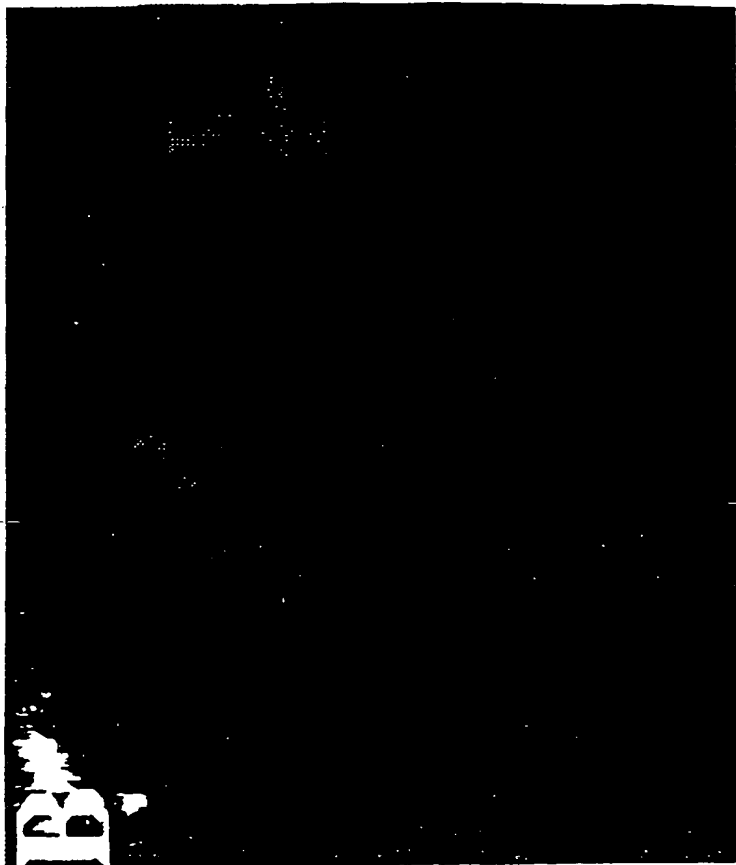


Figure 12B

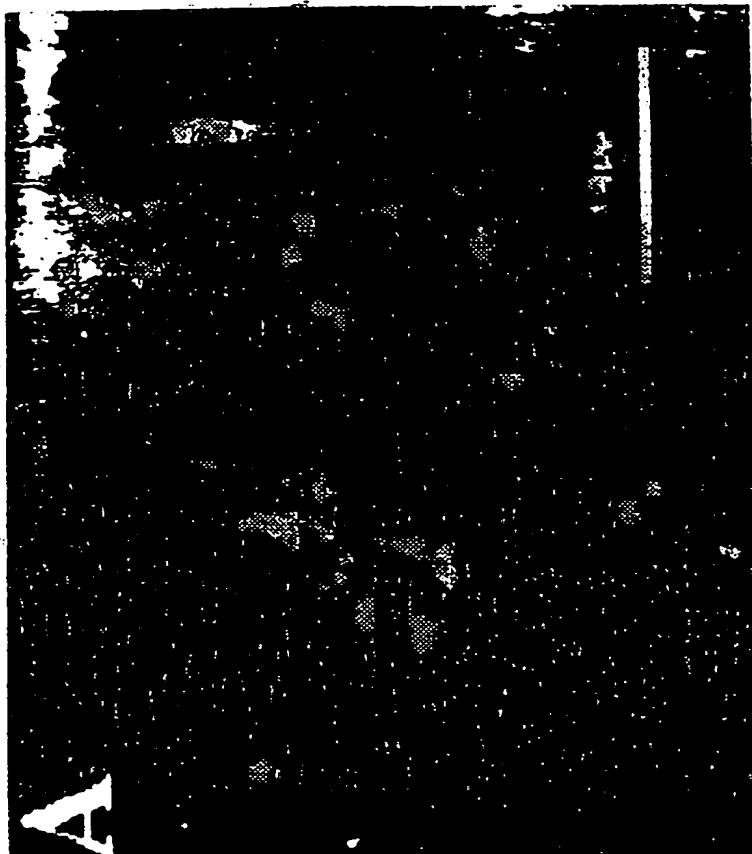


Figure 12A

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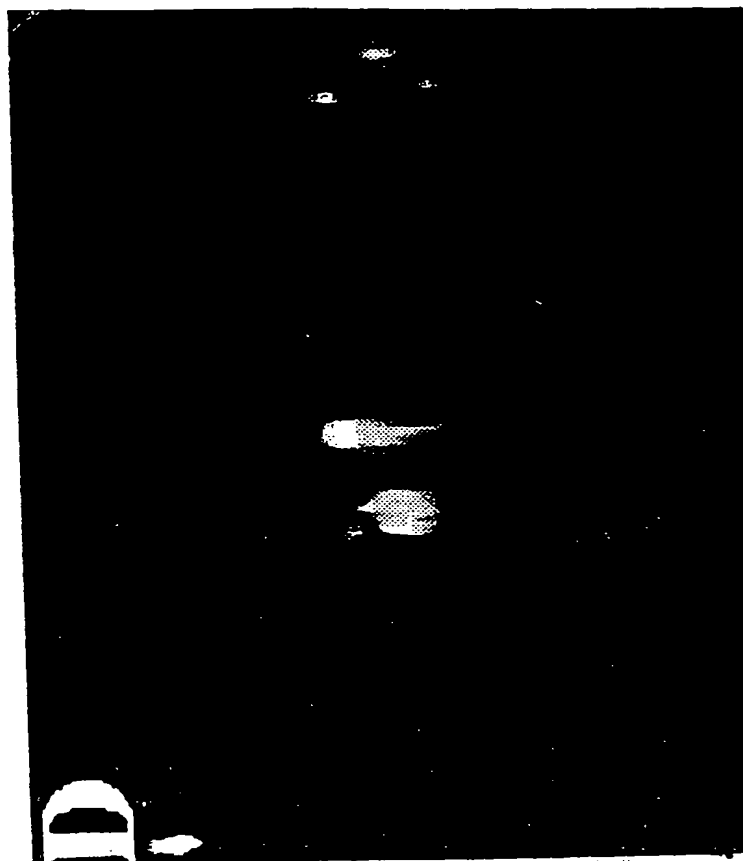


Figure 12D

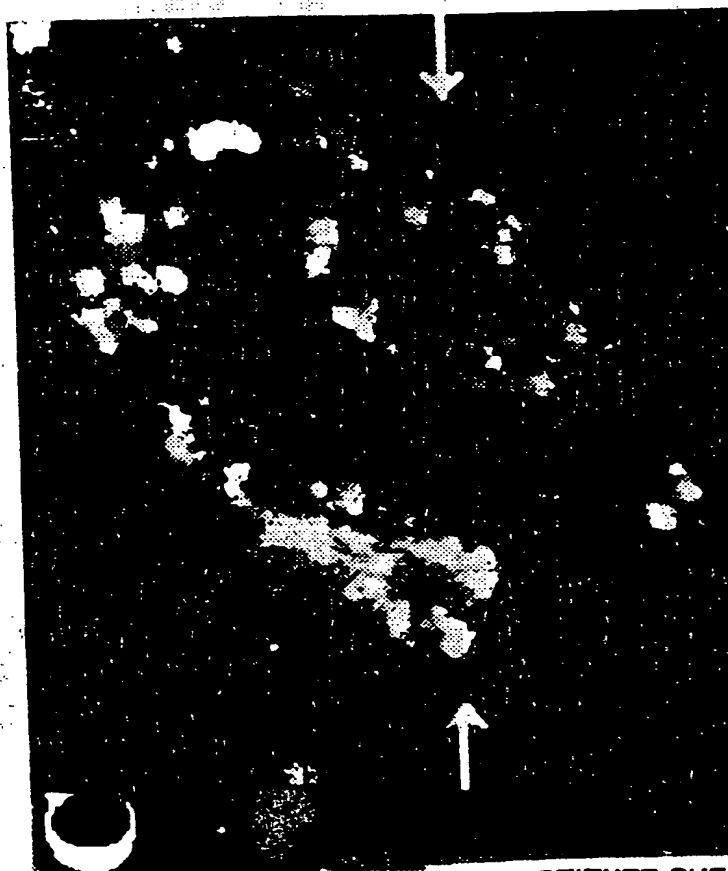


Figure 12C

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rec700 (WT), Baf⁻

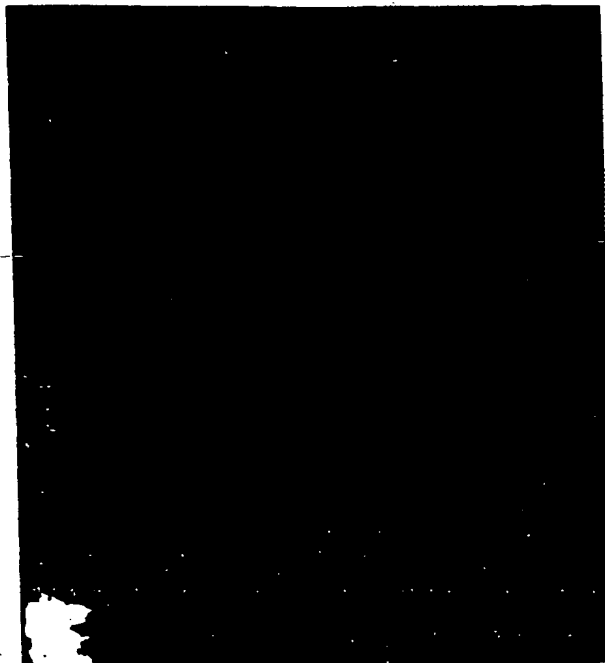


Figure 13B

rec700 (WT), Baf⁺



Figure 13A

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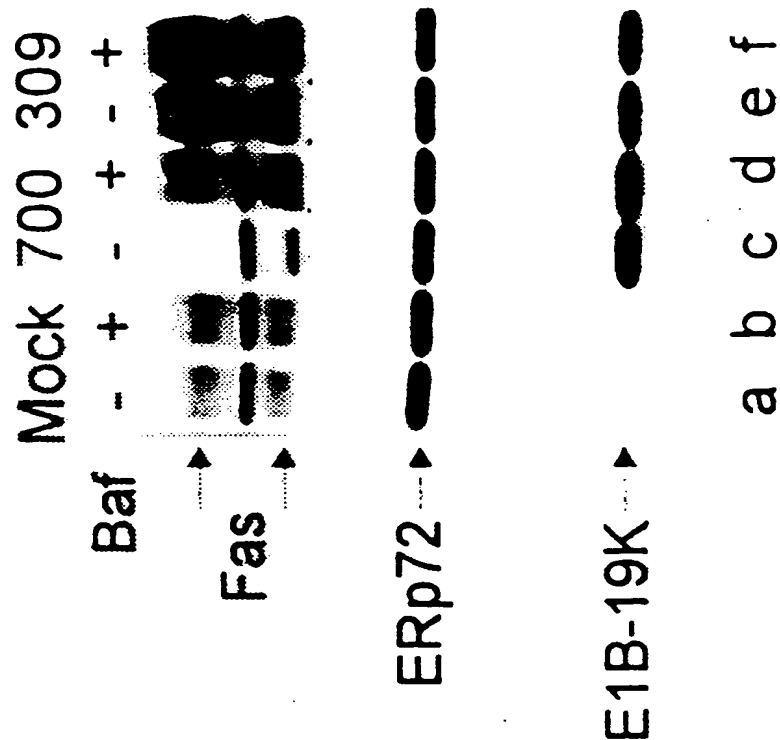


Figure 13D

d/309 (RID⁺), Baf⁺

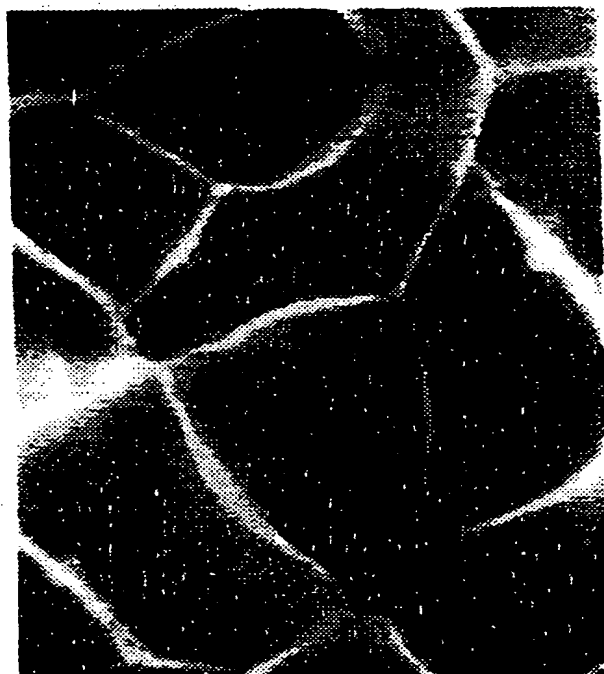


Figure 13C

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	Mock 700		309			
Baf	-	+	-	+	-	+
TfR	-----					

Figure 13E

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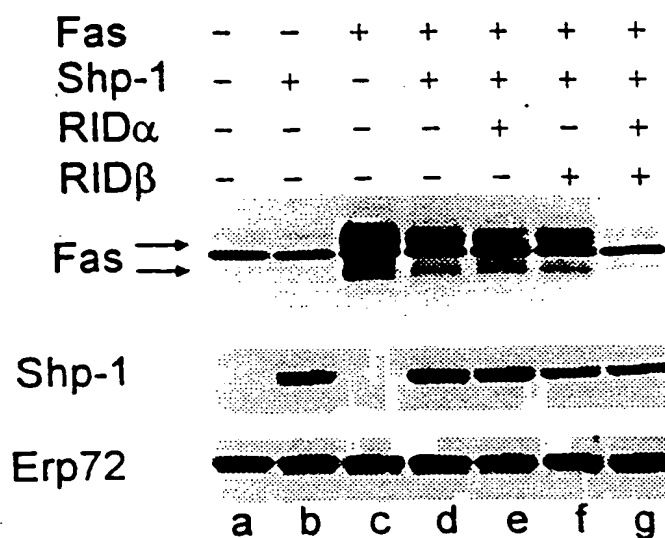


Figure 14

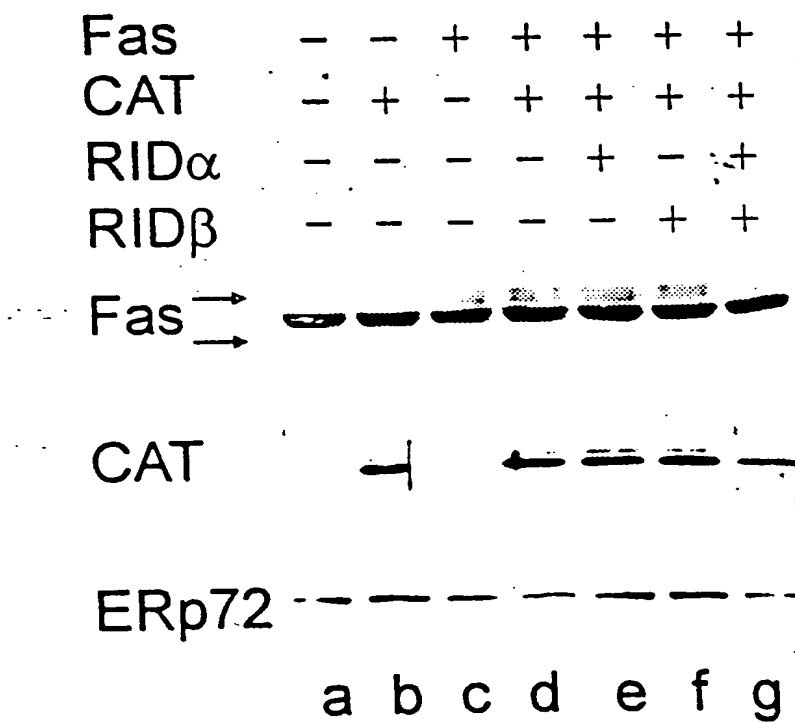


Figure 15

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Figure 16A

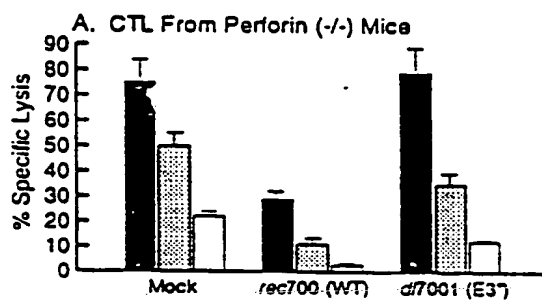


Figure 16B

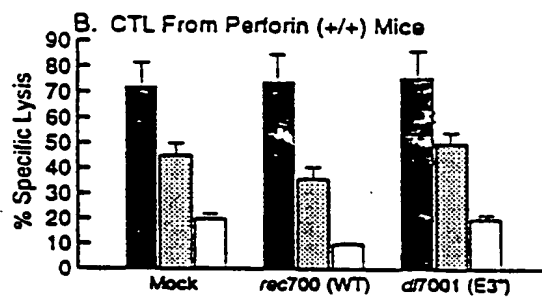
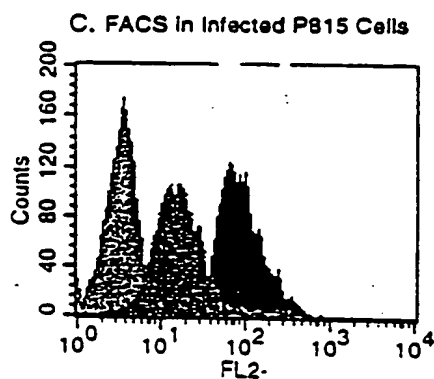


Figure 16C



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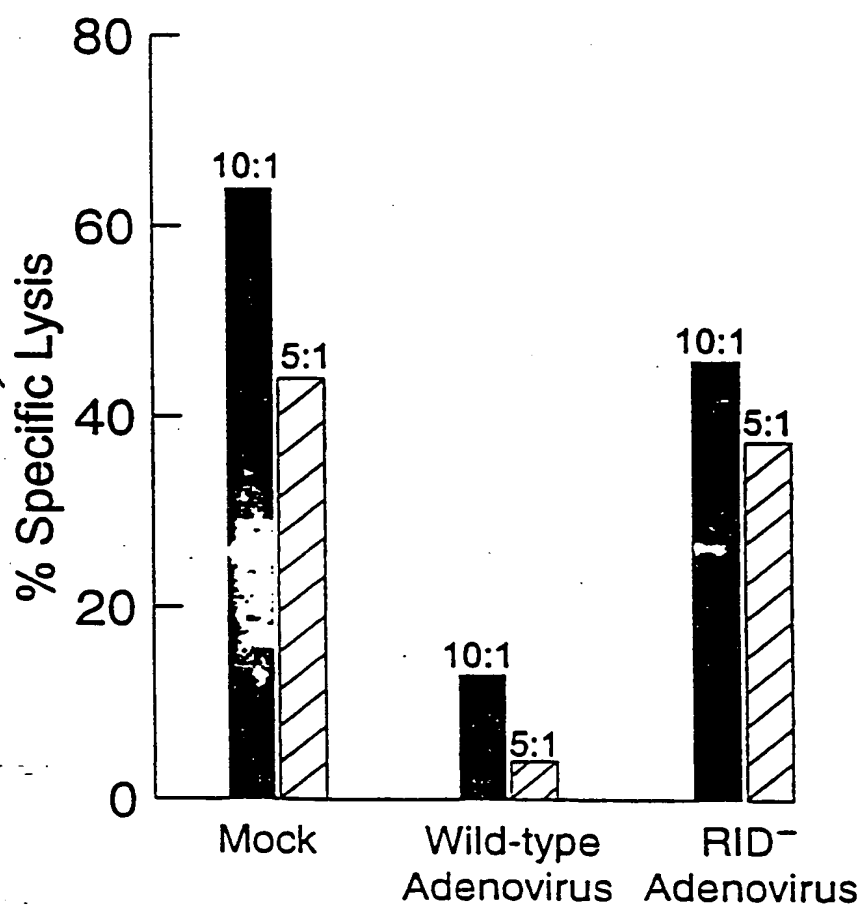
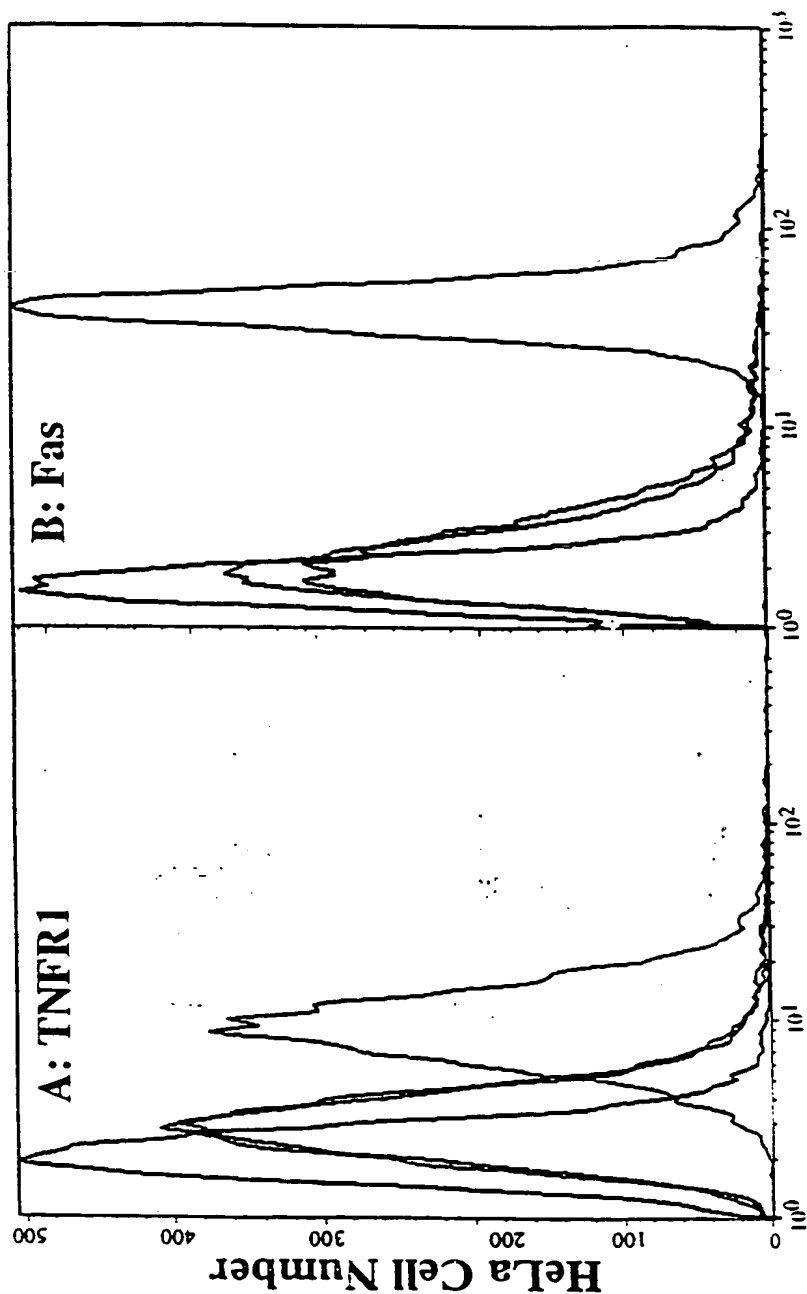


Figure 17

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Fluorescence Intensity

Mock - 100%
rec700 (Wild type) - 11%
dl712 (↑RID) - 14%
 Unstained control - 1.5%

Figure 18B

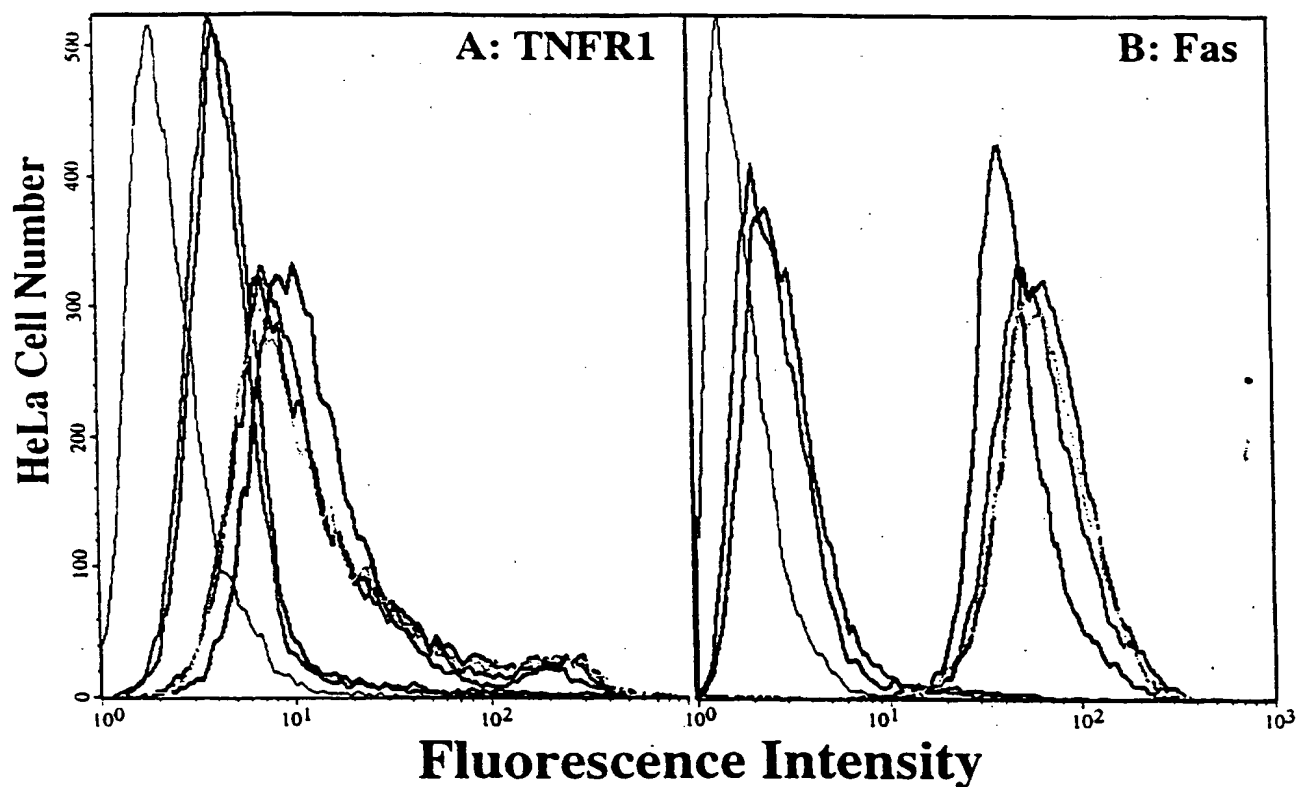
Mock - 93%
rec700 (Wild type) - 16%
dl712 (↑RID) - 18%
 Unstained control - 1.5%

Figure 18A

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Figure 19A

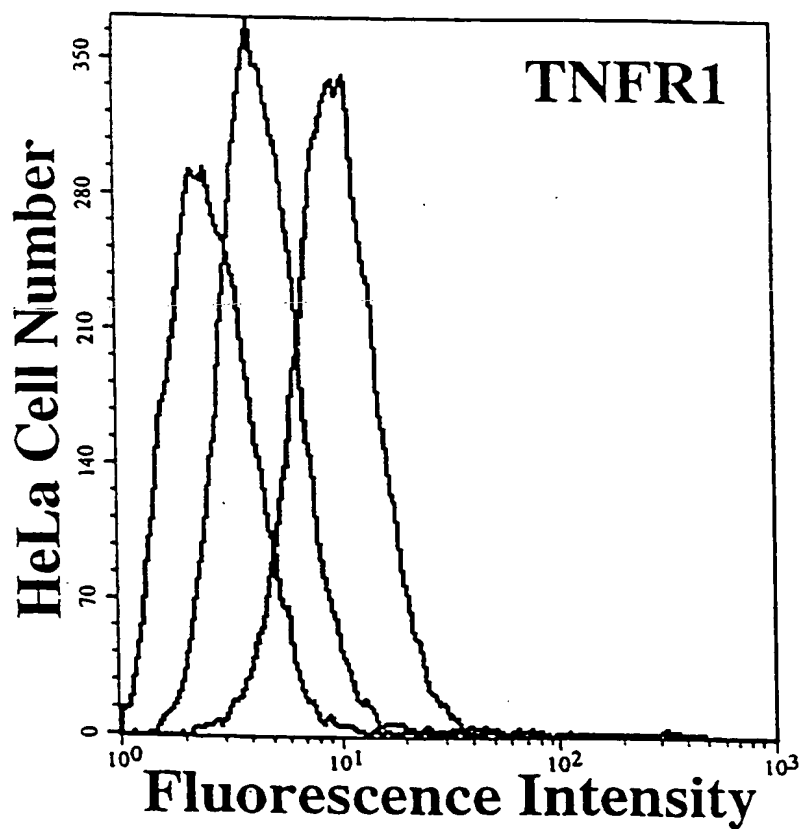
Figure 19B



Mock - 92%
rec700 (Wild type) - 29%
dl753 (RID α^{-}) - 85%
dl764 (RID β^{-}) - 84%
dl712 (\uparrow RID) - 24%
dl309 (RID $^{-}$) - 84%
 Unstained Control - 2%

Mock - 100%
rec700 (Wild type) - 4%
dl753 (RID α^{-}) - 100%
dl764 (RID β^{-}) - 100%
dl712 (\uparrow RID) - 2%
dl309 (RID $^{-}$) - 100%
 Unstained Control - 1%

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Mock - 93%

231-10 (E3⁺ vector) 24 hr. p.i. - 35%

231-10 (E3⁺ vector) 48hr. p.i. - 11%

Figure 20

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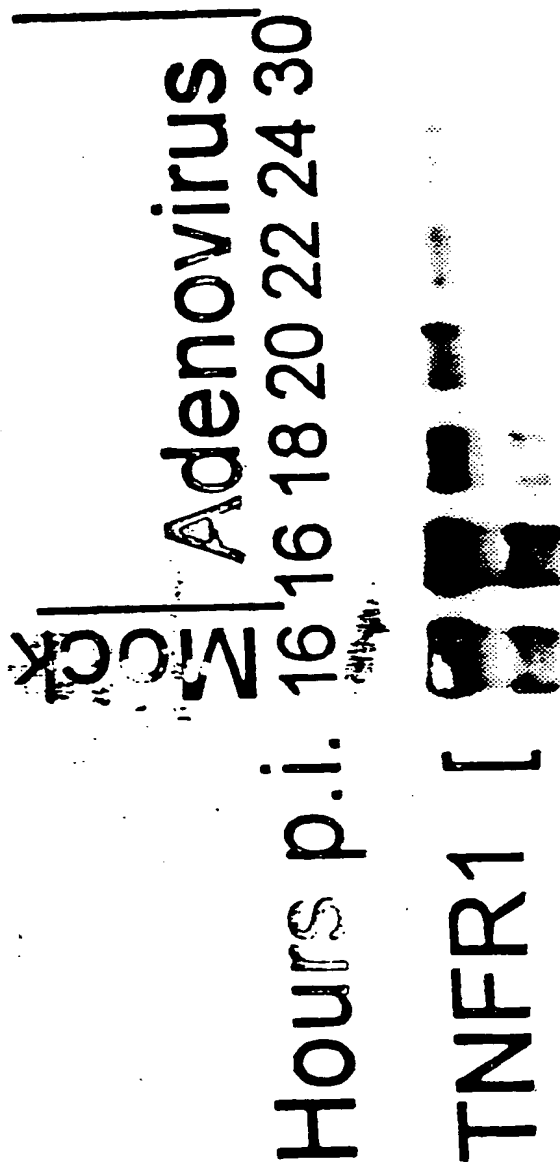


Figure 21

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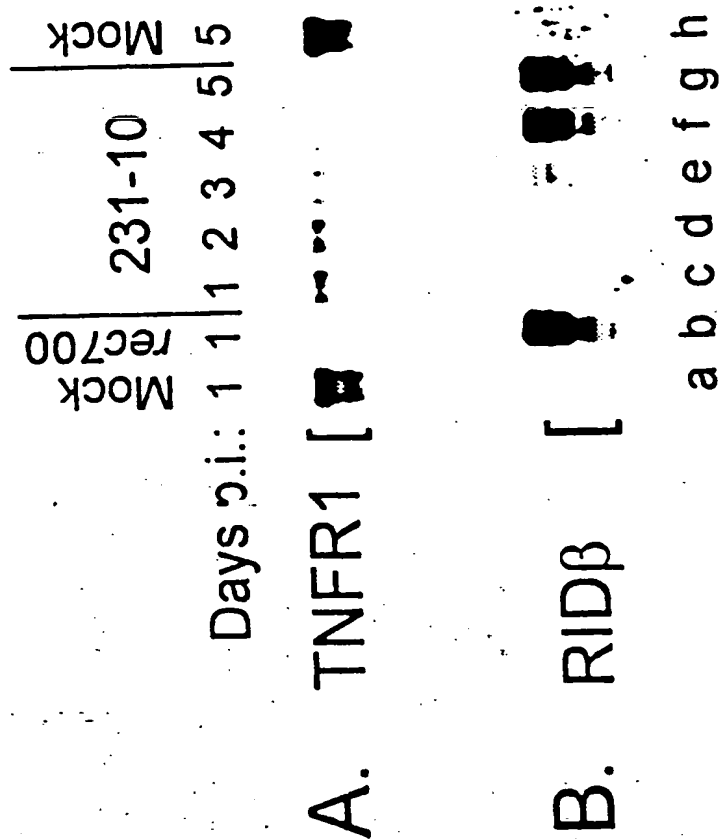


Figure 22

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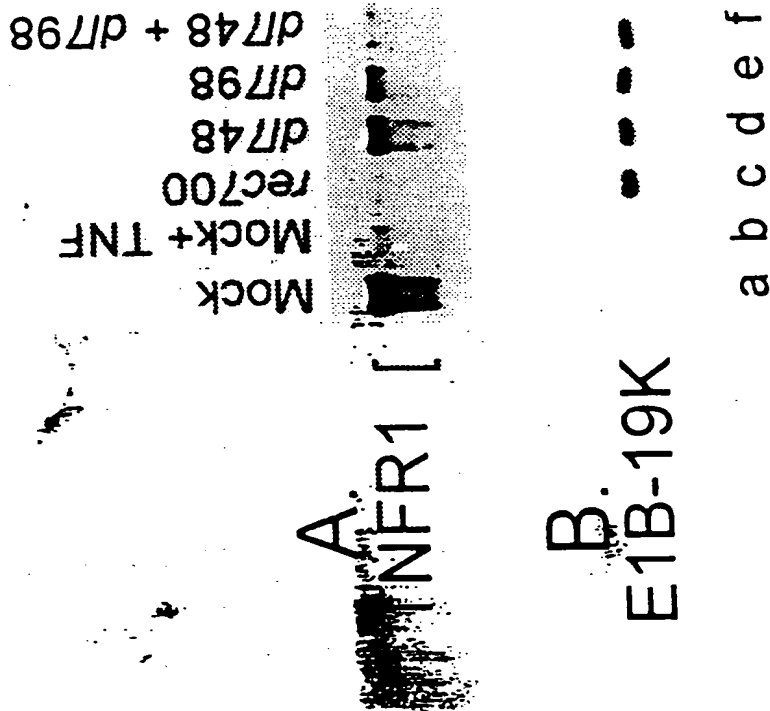


Figure 23

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Figure 24

31/85



Figure 25

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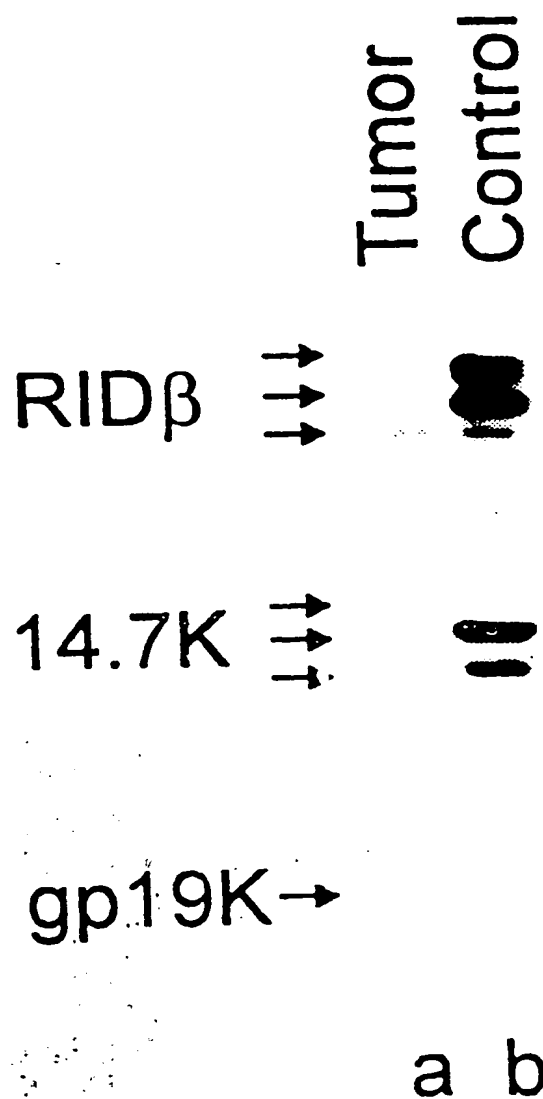


Figure 26

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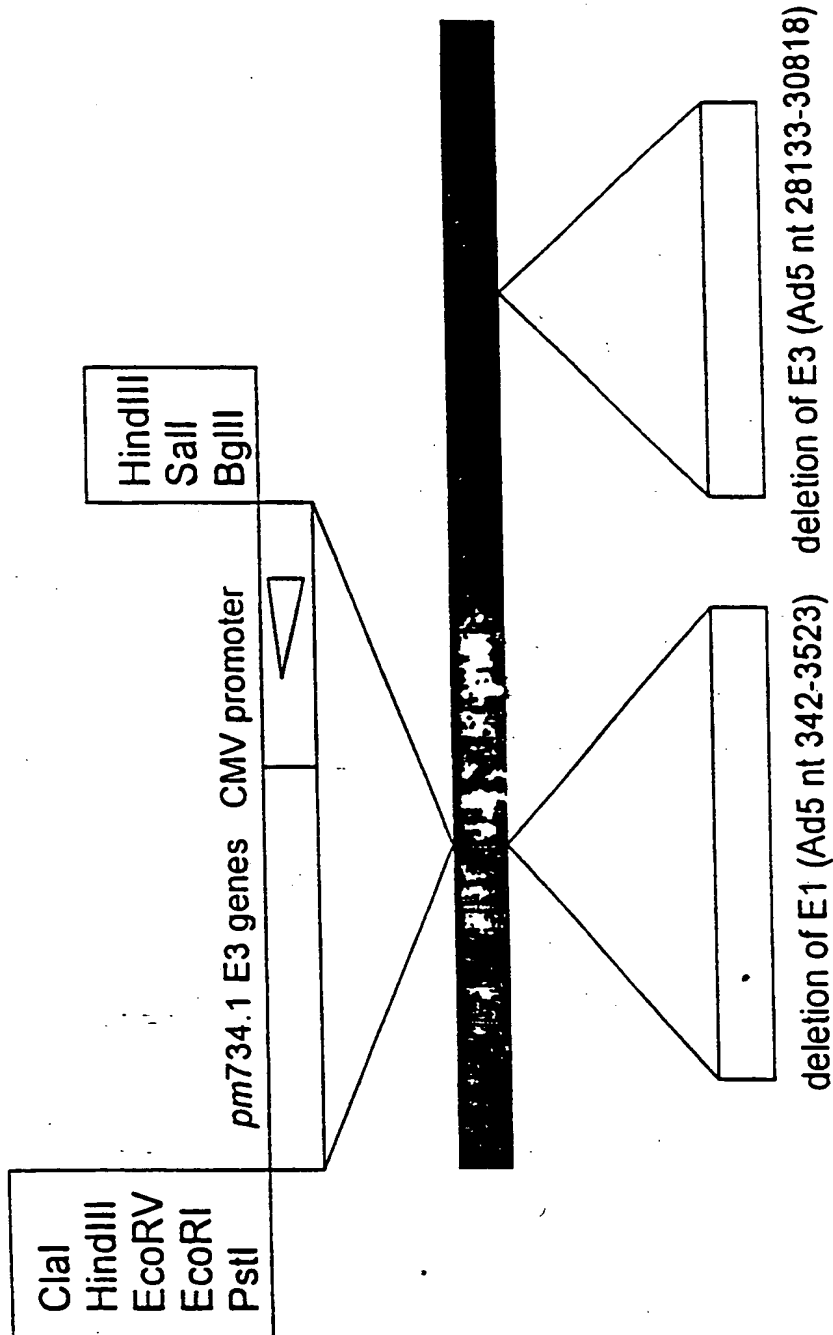


Figure 27

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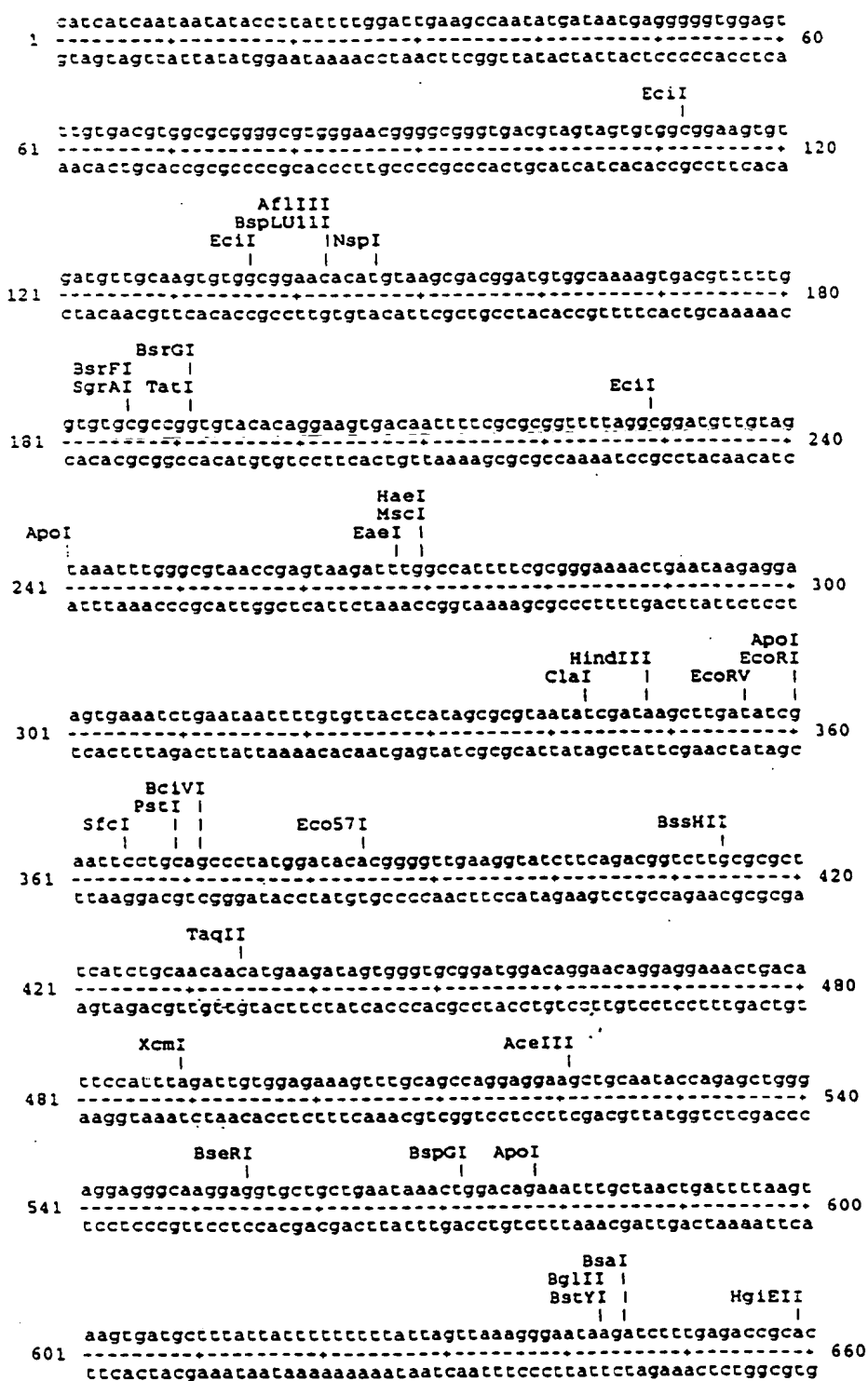


Figure 28A

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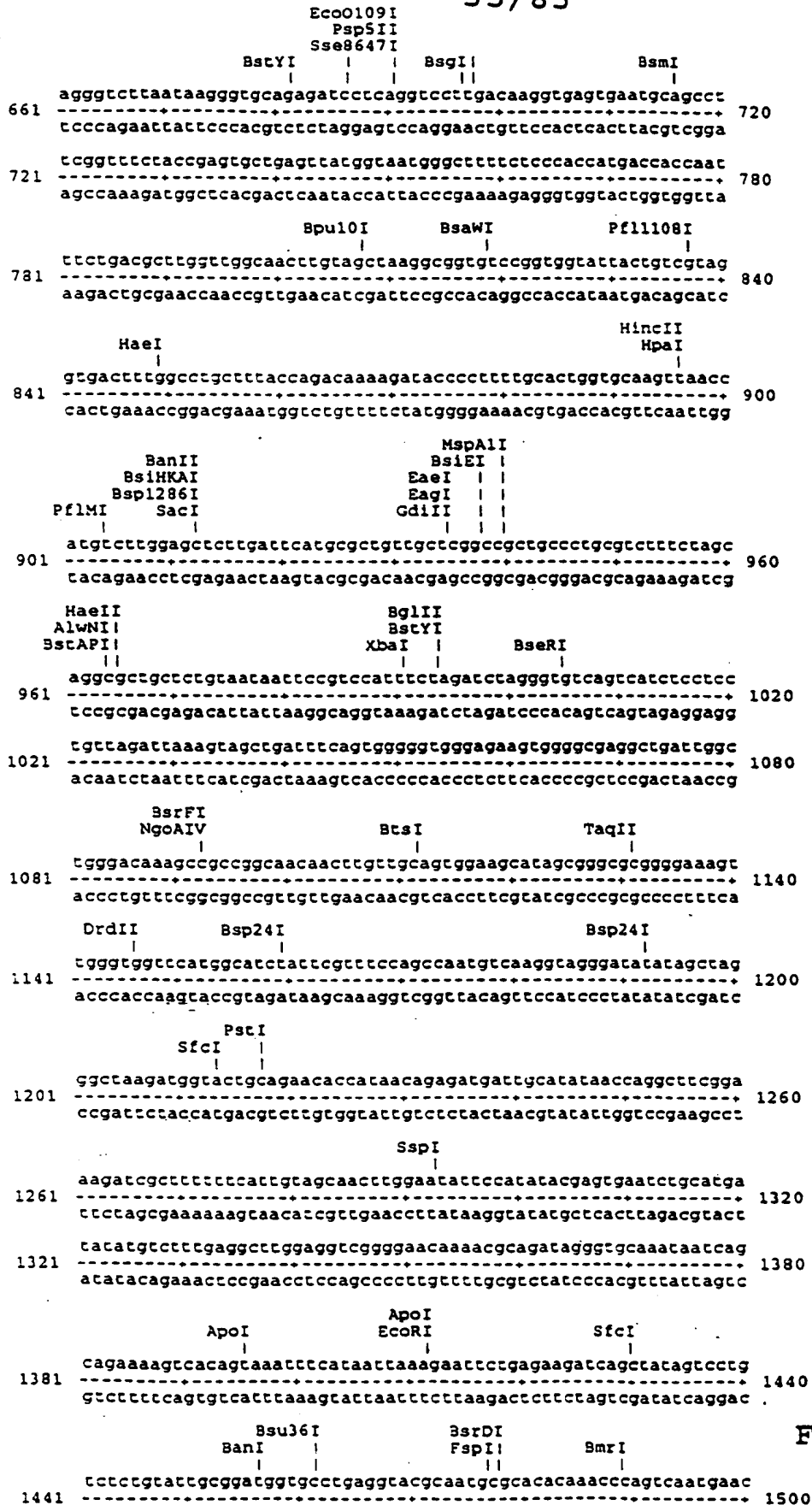


Figure 28B

36/85

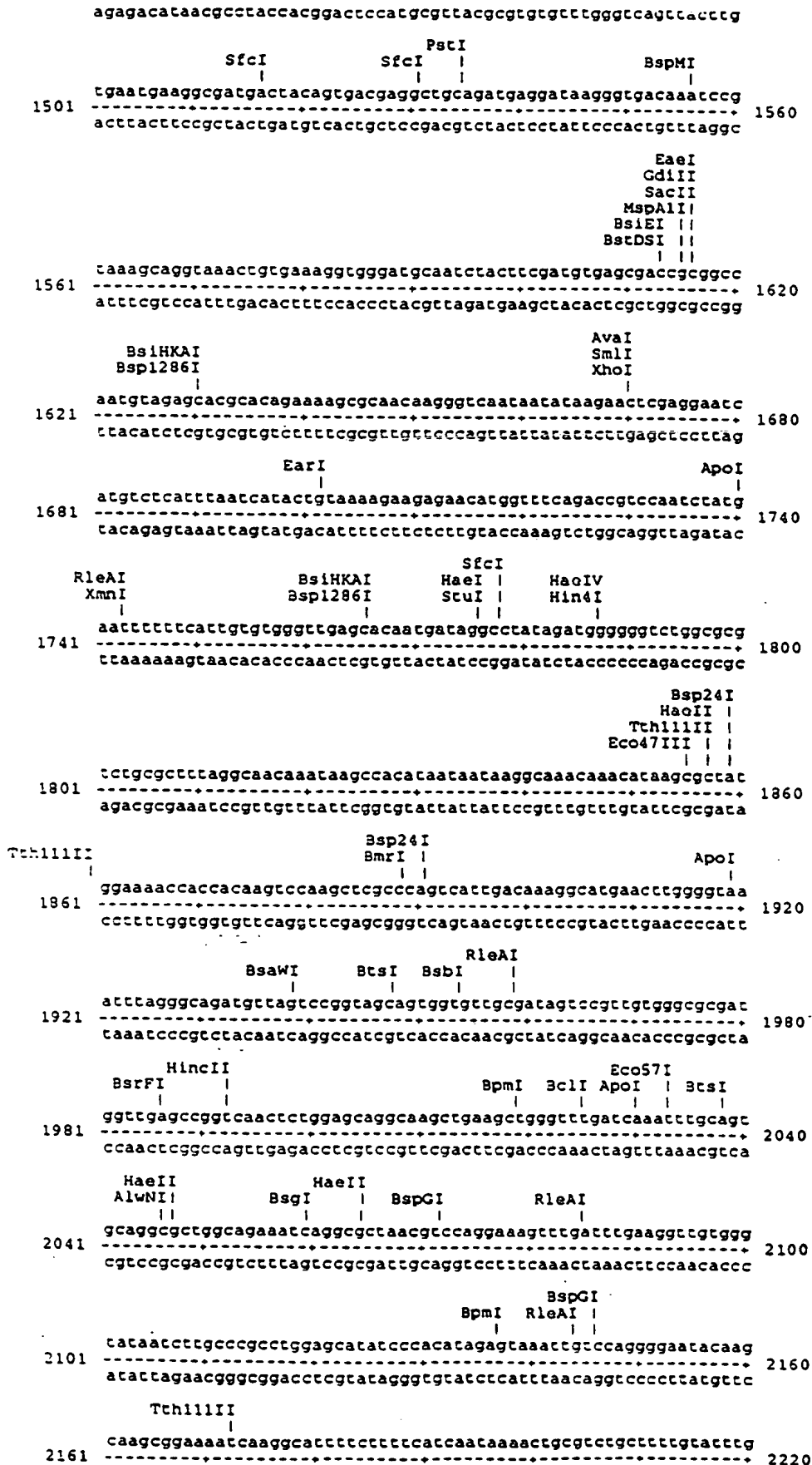


Figure 28C

37/85

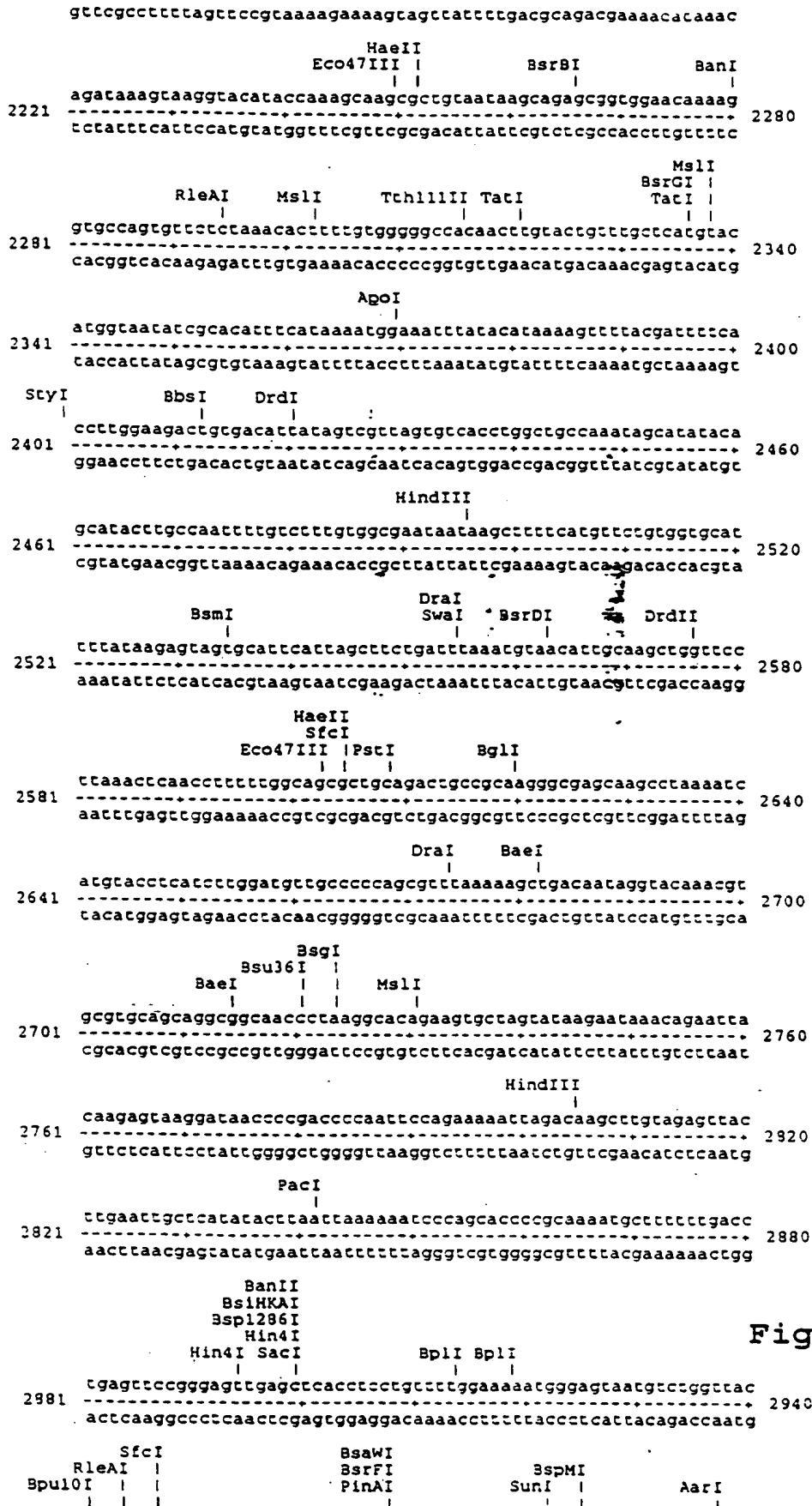


Figure 28D

38/85

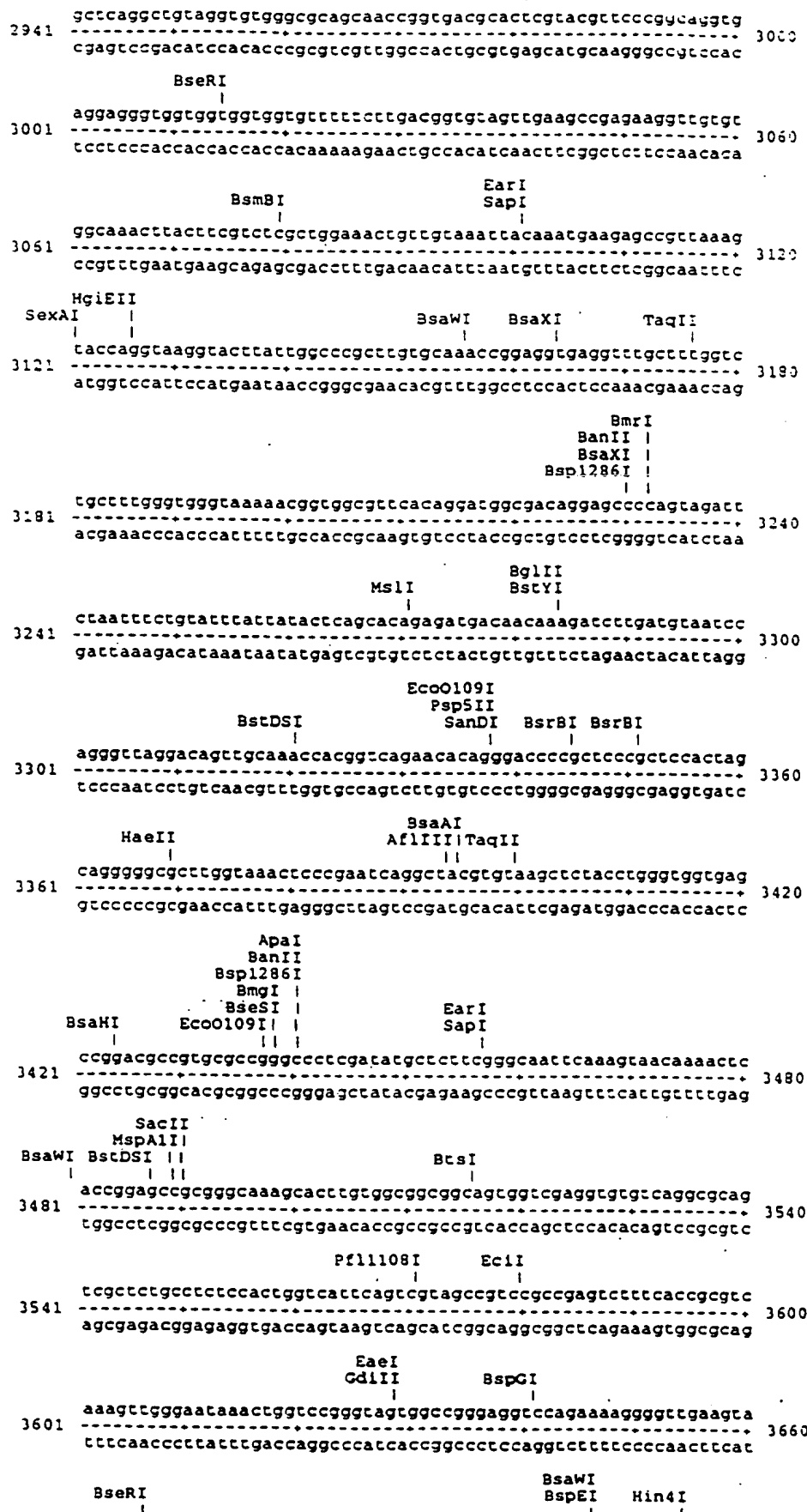


Figure 28E

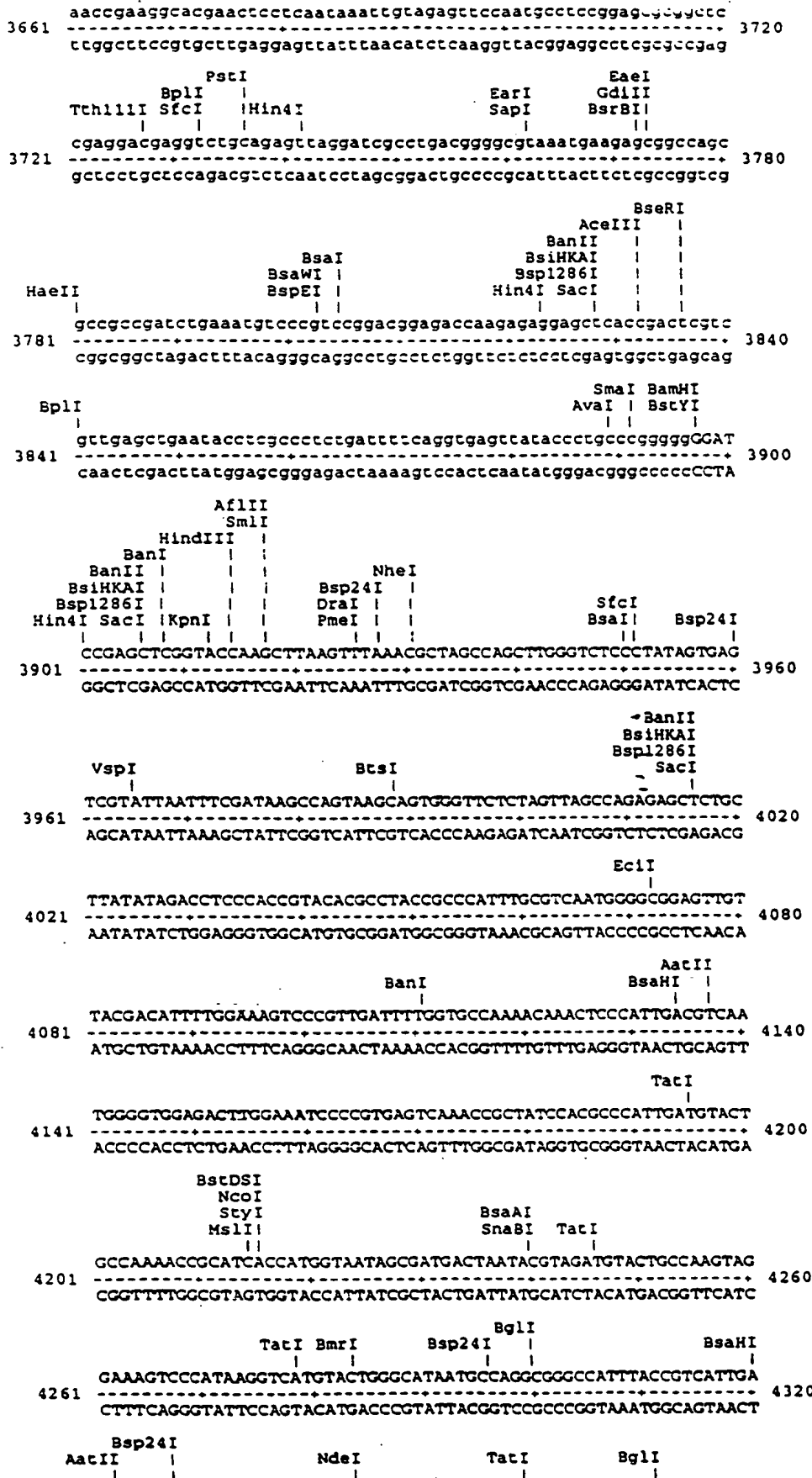


Figure 28F

40/85

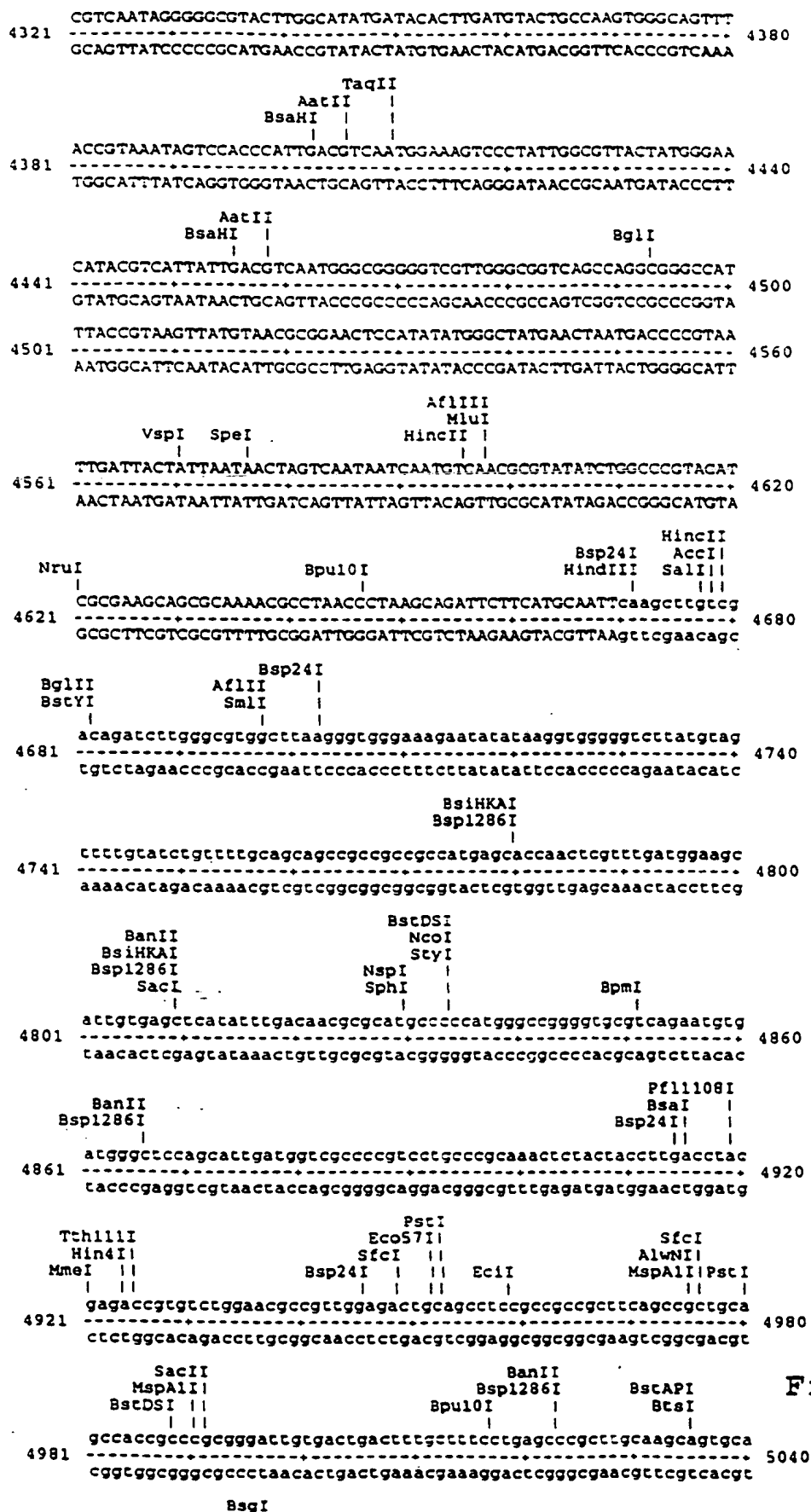


Figure 28G

[illegible]

Figure 28H

42/85

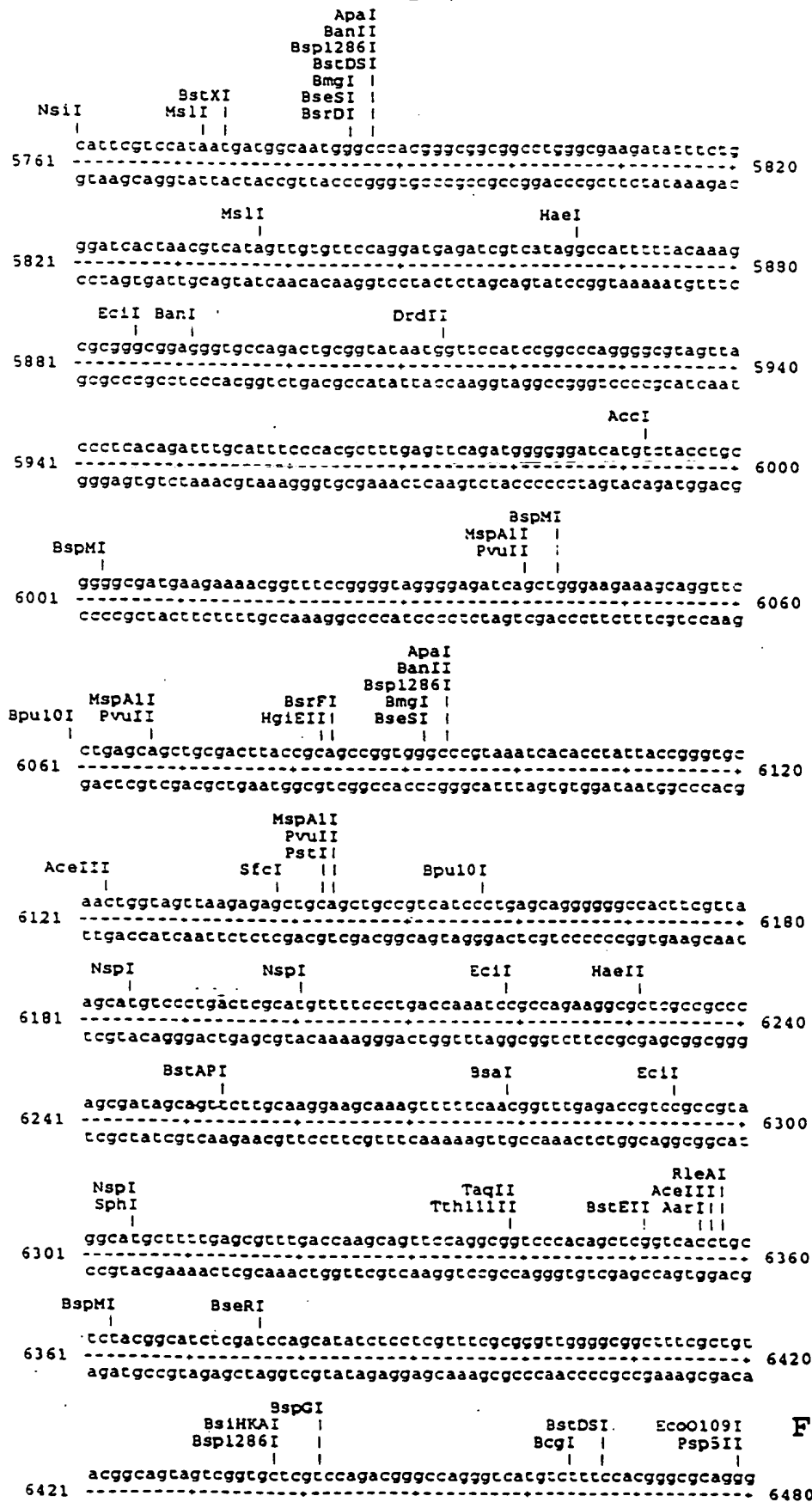


Figure 28I

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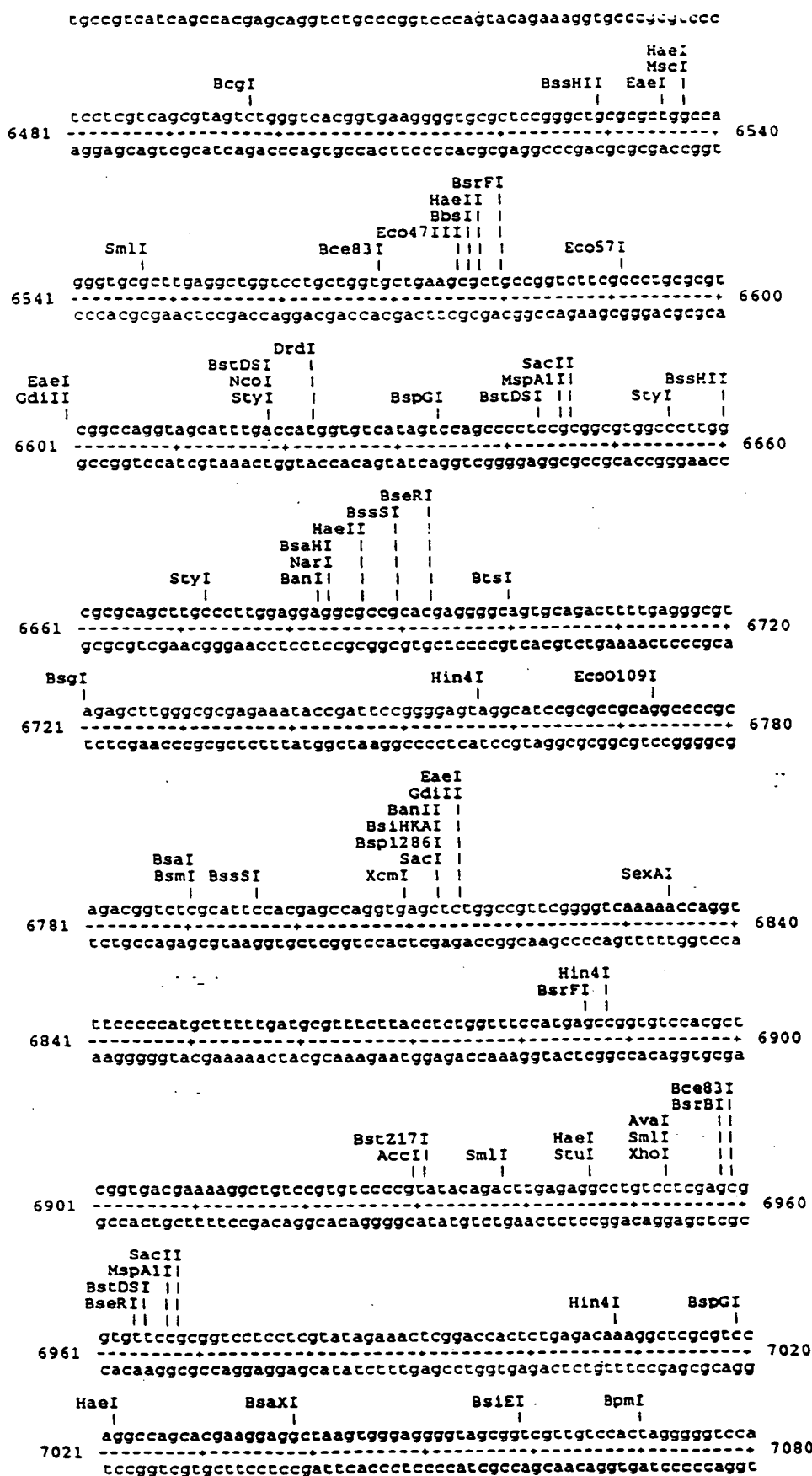


Figure 28J

44/85

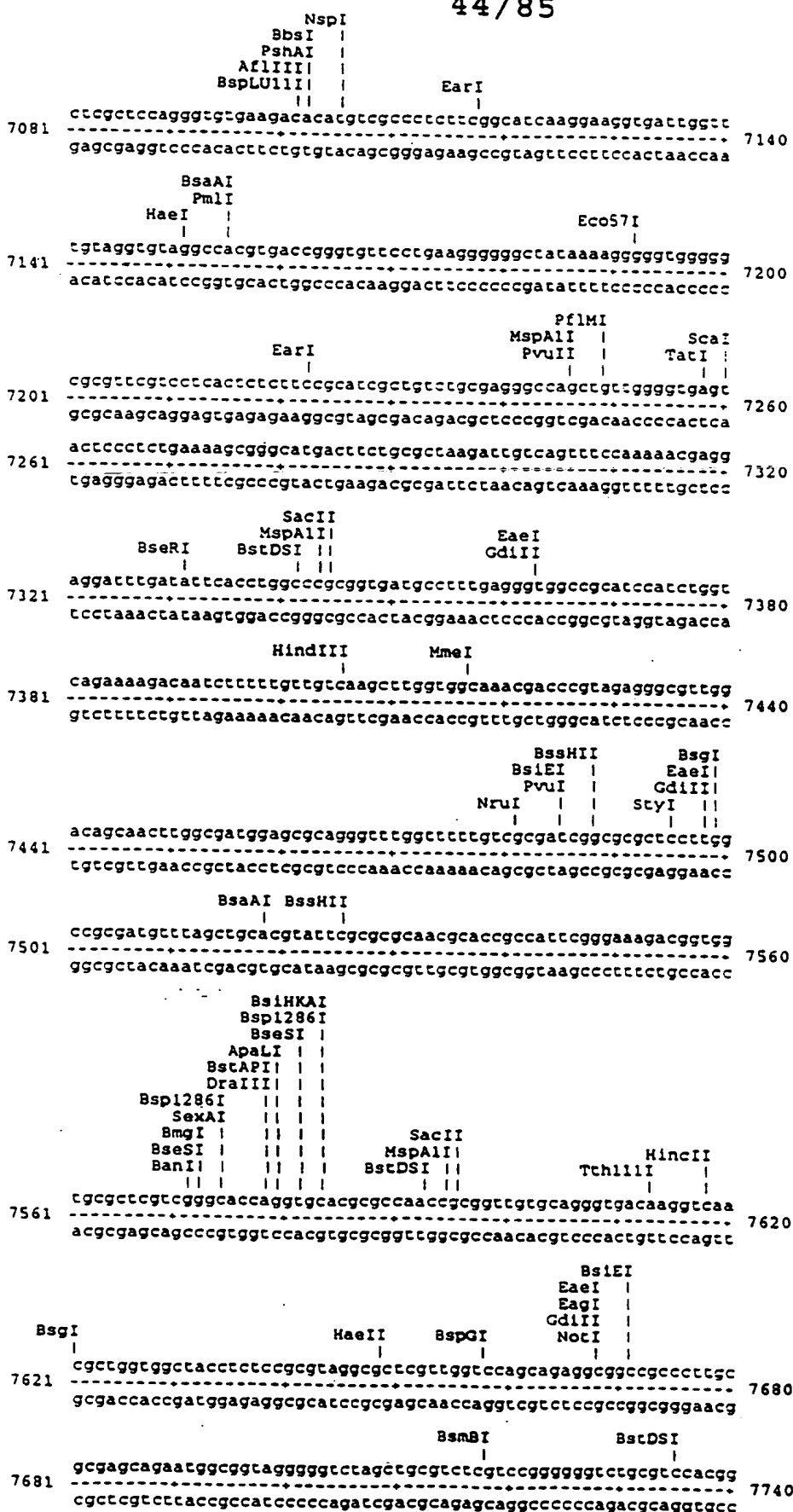


Figure 28K

45/85

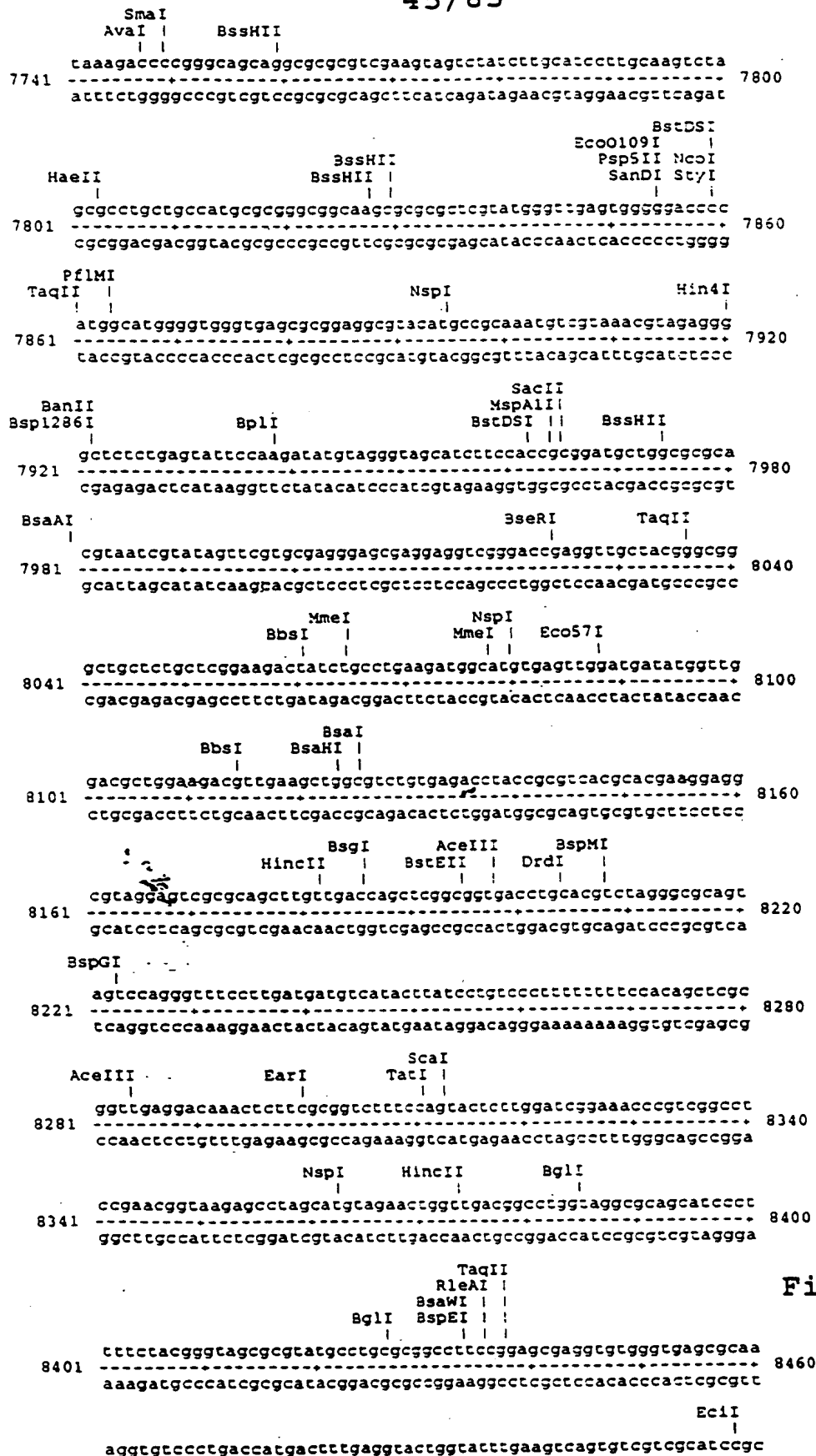


Figure 28L

46/85

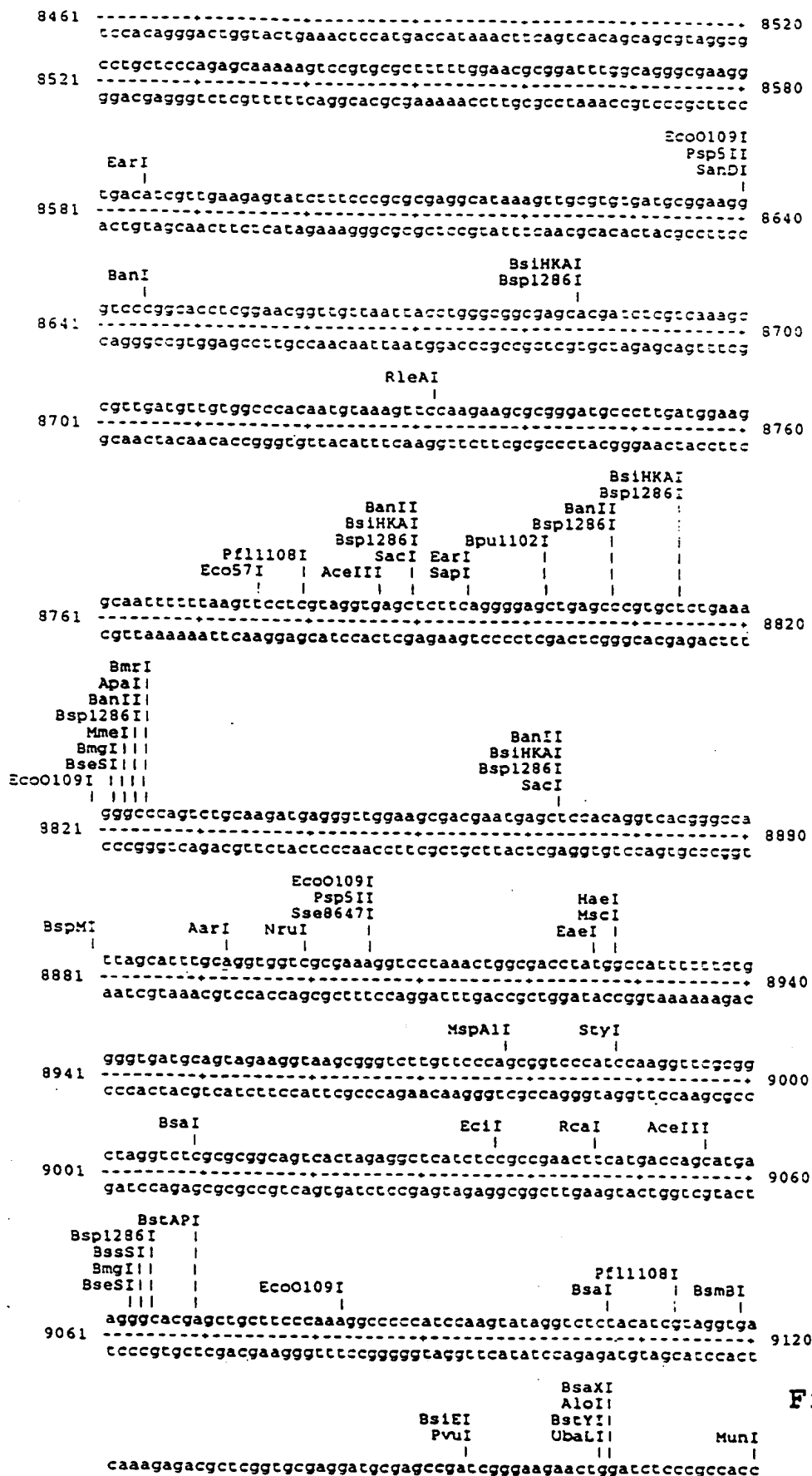


Figure 28M

47/85

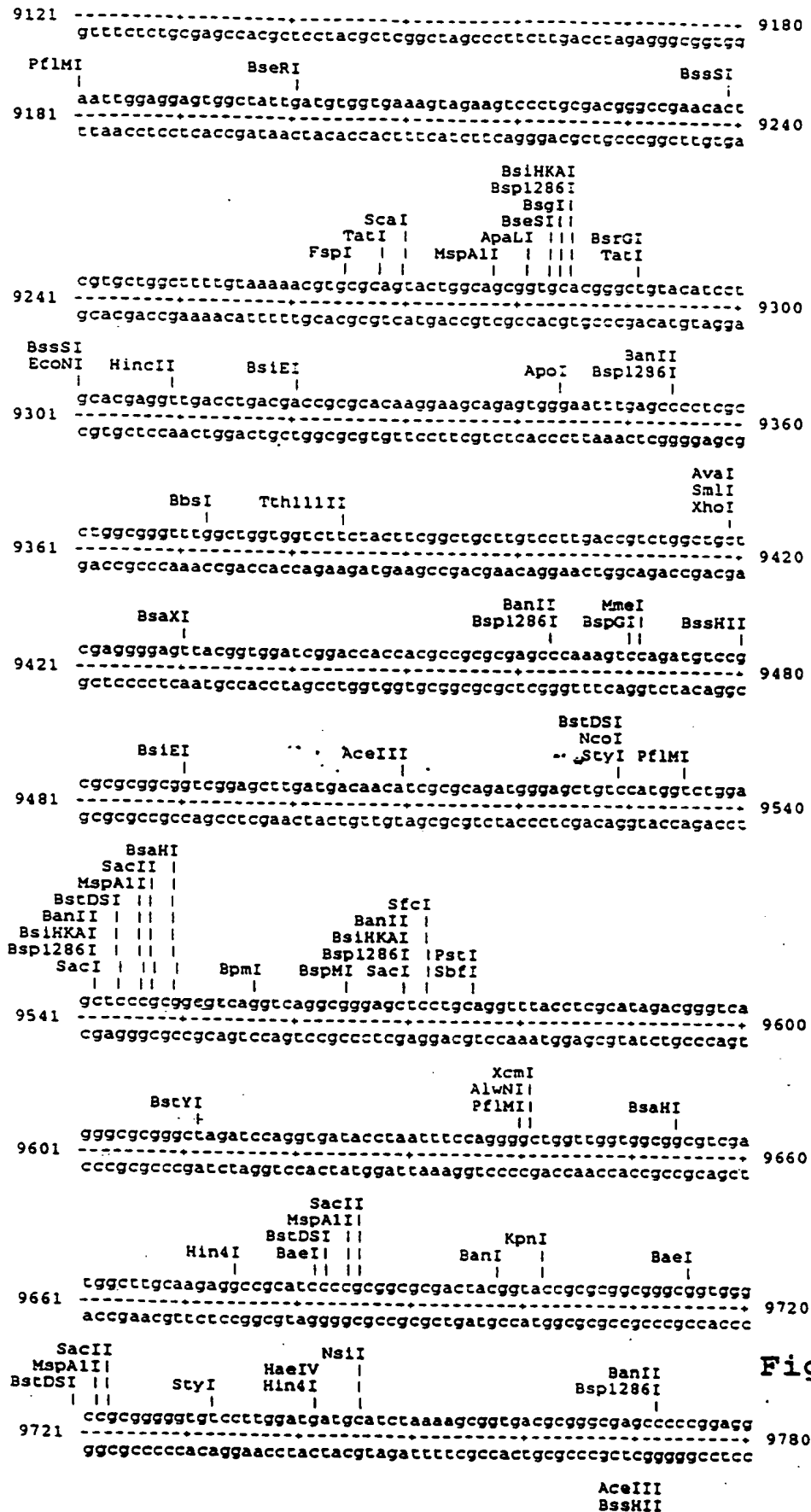


Figure 28N

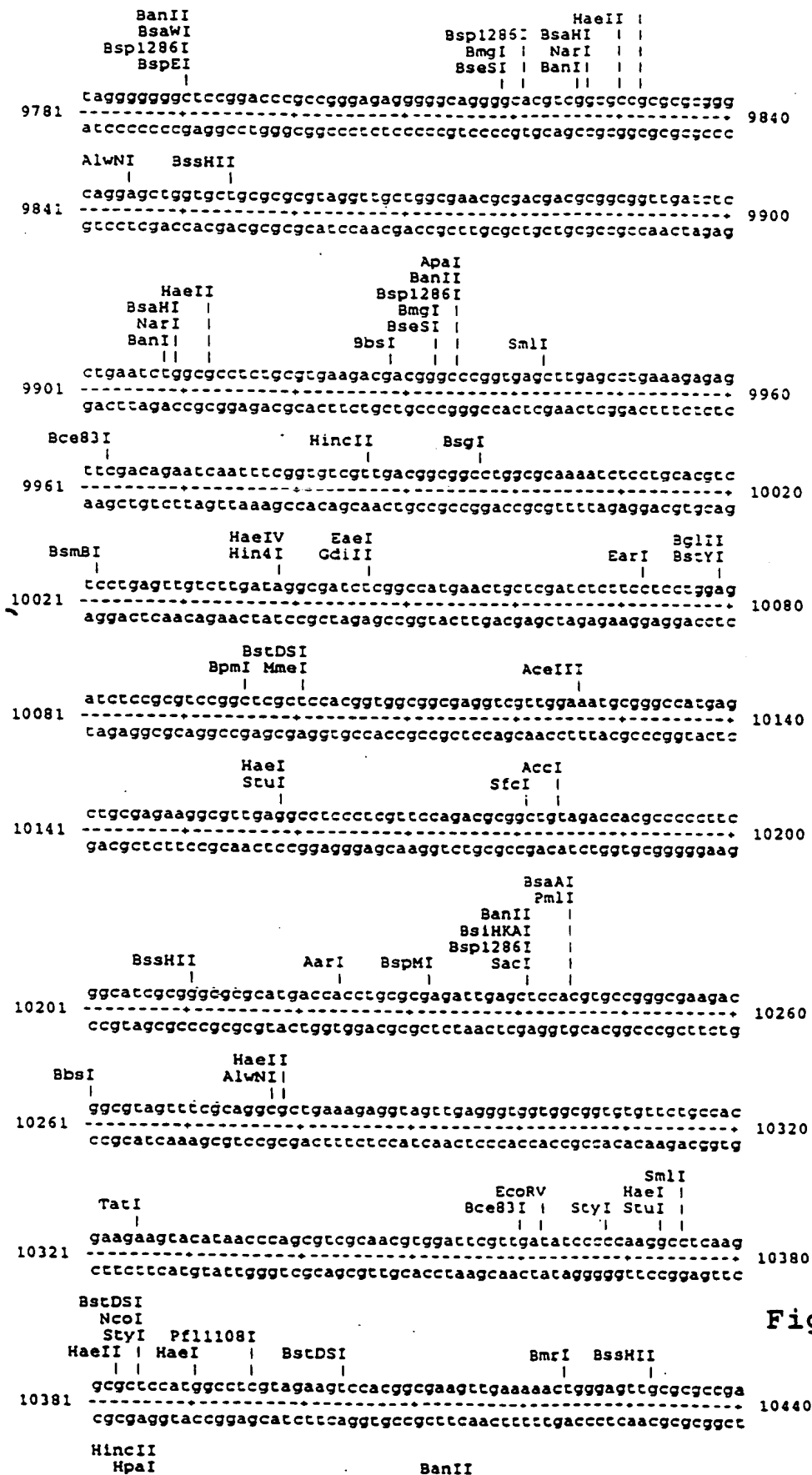


Figure 280

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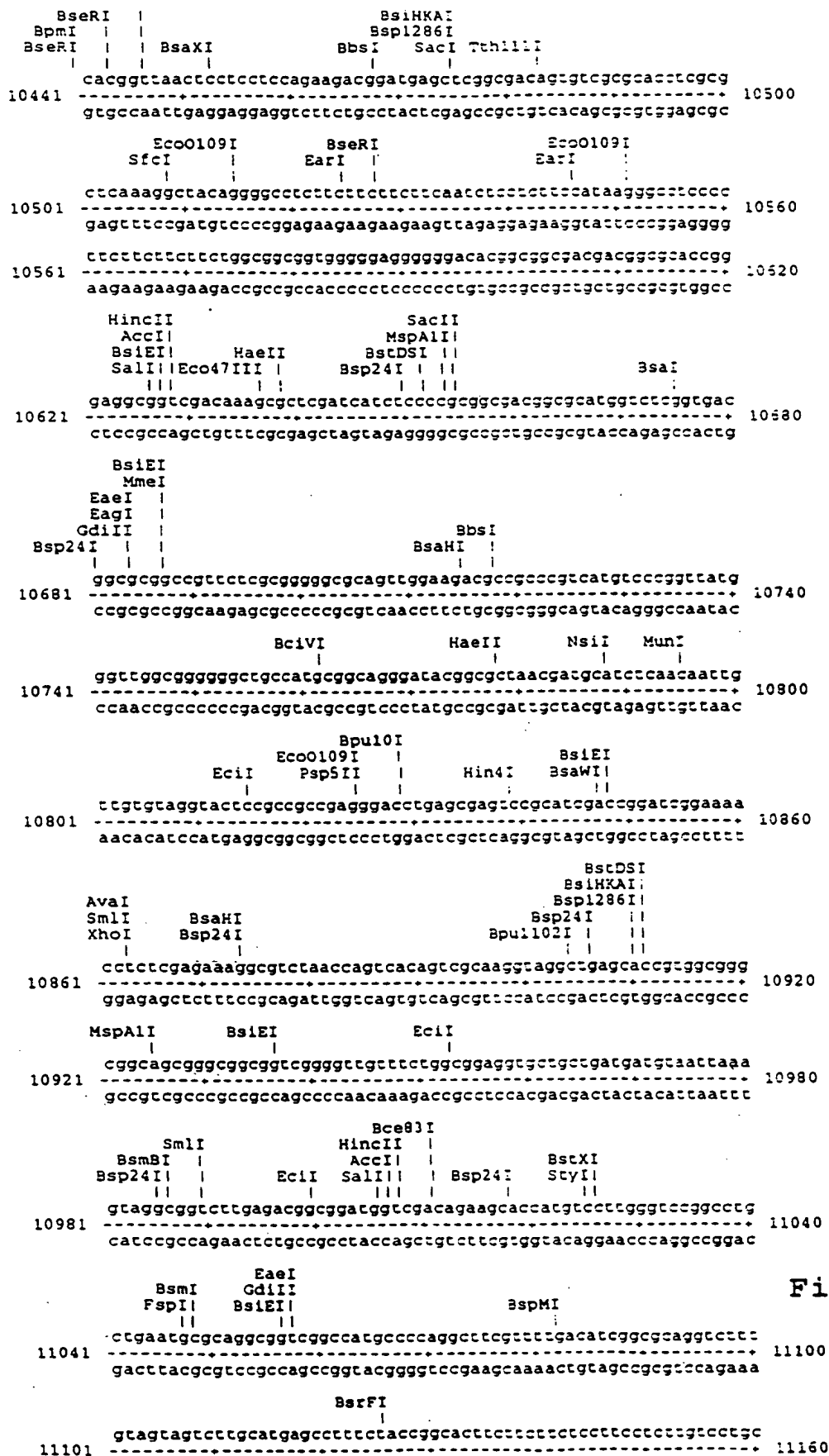


Figure 28P

50/85

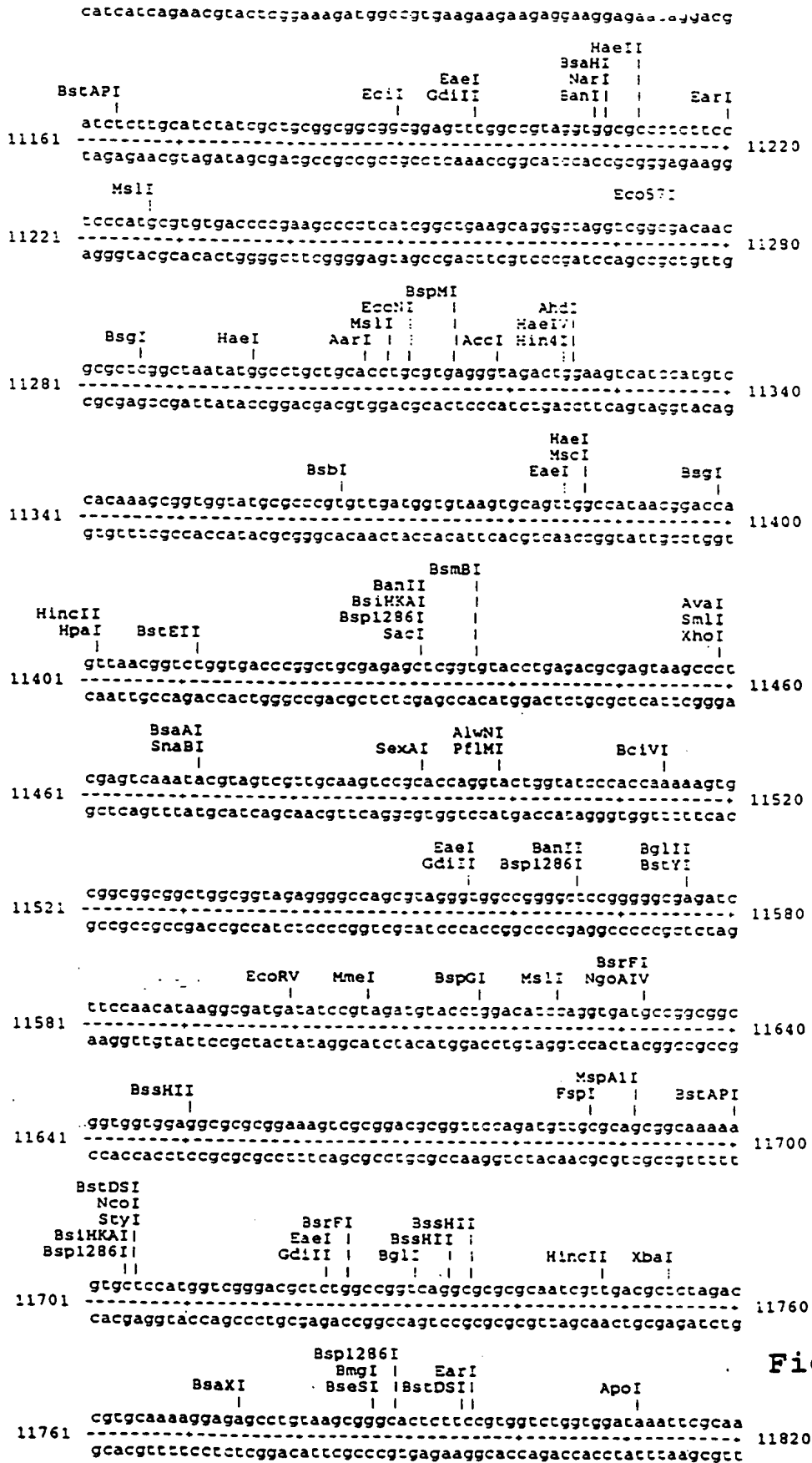


Figure 28Q

11821

11880

11881

11940

11941

12000

12001

12060

12061

12120

12121

12180

12181

12240

12241

12300

12301

12360

12361

12420

12421

12480

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12660

12661

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12721

12780

12781

12840

12841

12900

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12960

12961

13020

13021

13080

13081

13140

13141

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13201

13260

13261

13320

13321

13380

13381

13440

13441

13500

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13561

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15061

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15121

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15181

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15301

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16081

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16200

16201

16260

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16320

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16380

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16440

16441

16500

16501

16560

16561

16620

16621

16680

16681

16740

16741

16800

16801

16860

16861

16920

16921

16980

16981

17040

17041

17100

17101

17160

17161

17220

17221

17280

17281

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17400

17401

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17461

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17640

17641

17700

17701

17760

17761

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17880

17881

17940

17941

18000

18001

18060

18061

18120

18121

18180

18181

18240

18241

18300

18301

18360

18361

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18481

18540

18541

18600

18601

18660

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18720

18721

18780

18781

18840

18841

18900

18901

18960

18961

19020

19021

19080

19081

19140

19141

19200

19201

19260

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19321

19380

19381

19440

19441

19500

19501

19560

19561

19620

19621

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19681

19740

19741

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19801

19860

19861

19920

19921

19980

19981

20040

20041

20100

20101

20160

20161

20220

20221

20280

20281

20340

20341

20400

20401

20460

20461

20520

20521

20580

20581

20640

20641

20700

20701

20760

20761

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20881

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21000

21001

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21121

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21181

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21241

21300

21301

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21361

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21480

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21601

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21661

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21841

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21901

21960

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22081

22140

22141

22200

22201

22260

22261

22320

22321

22380

22381

22440

22441

22500

22501

22560

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22741

22800

22801

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22861

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22980

22981

23040

23041

23100

23101

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23400

23401

23460

23461

23520

Figure 28R

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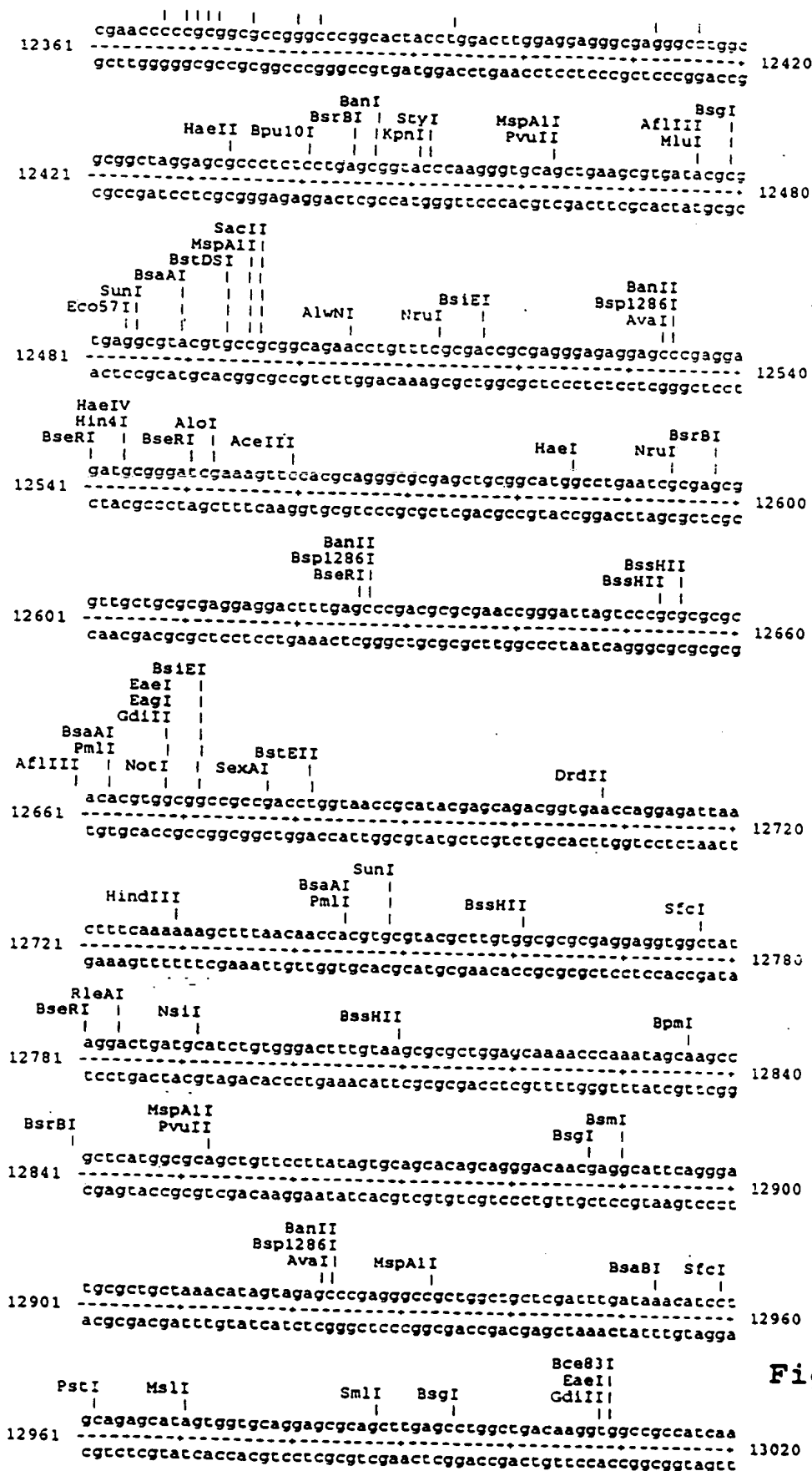


Figure 28S

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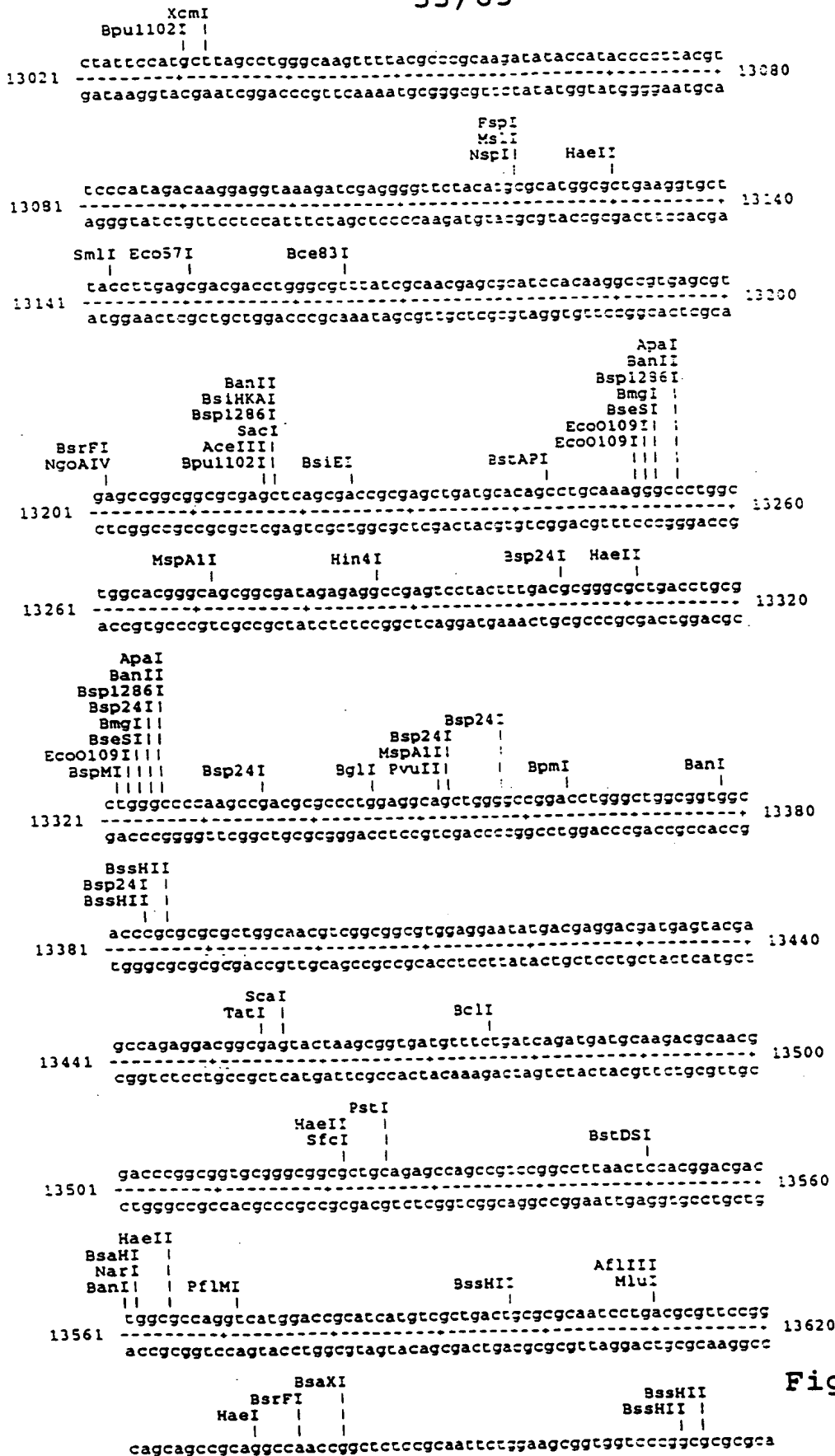


Figure 28T

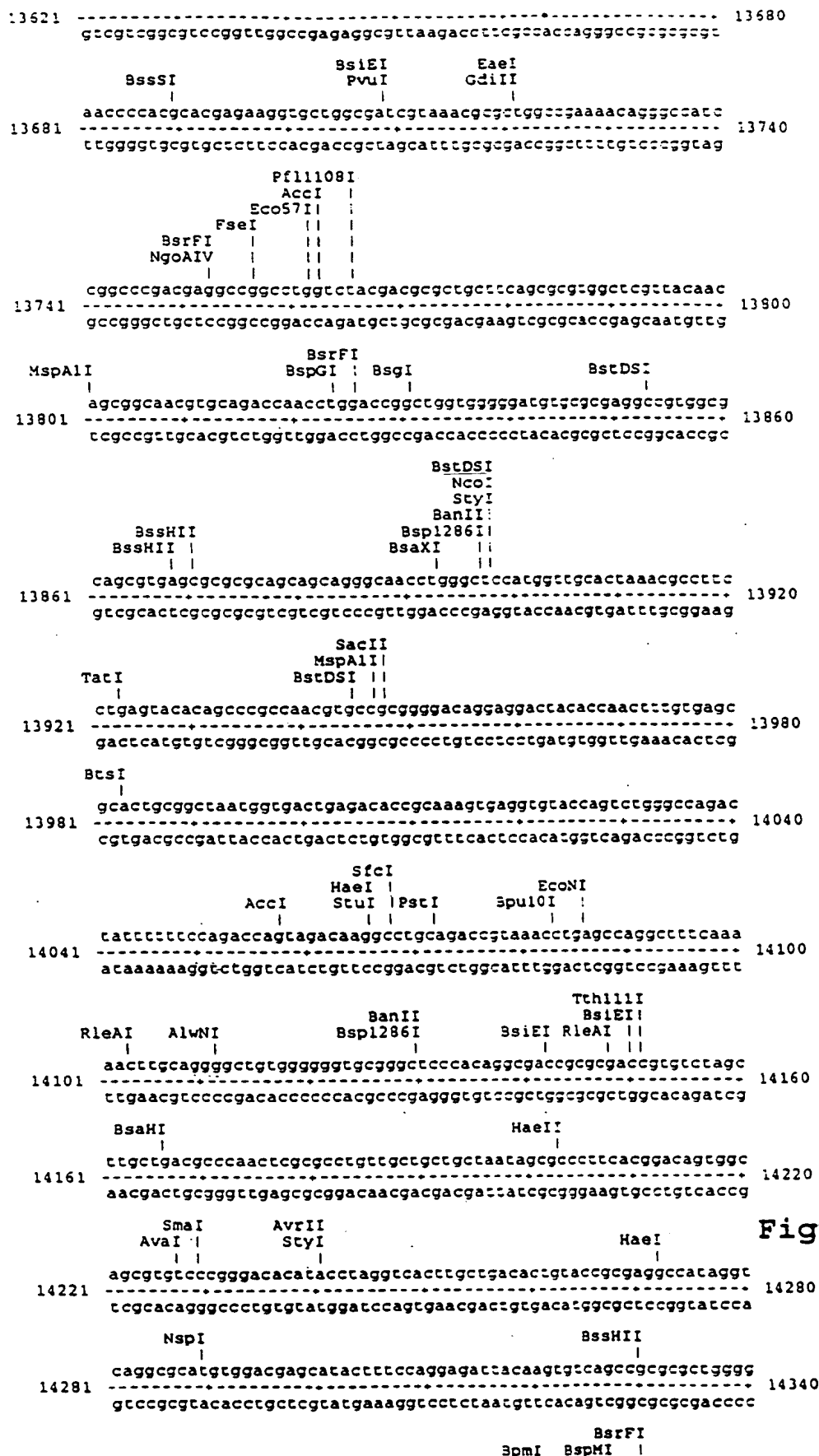


Figure 28U

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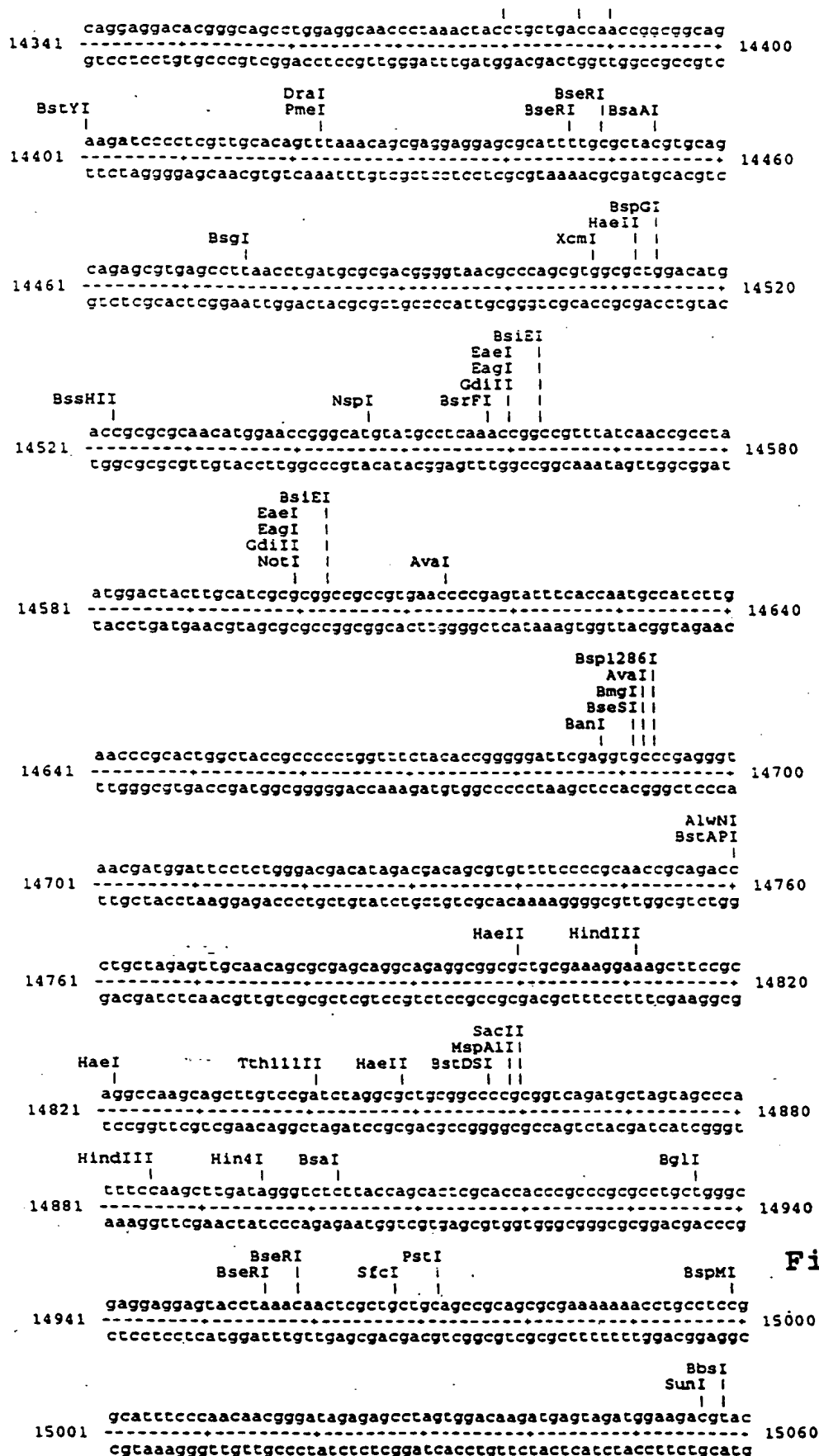


Figure 28V

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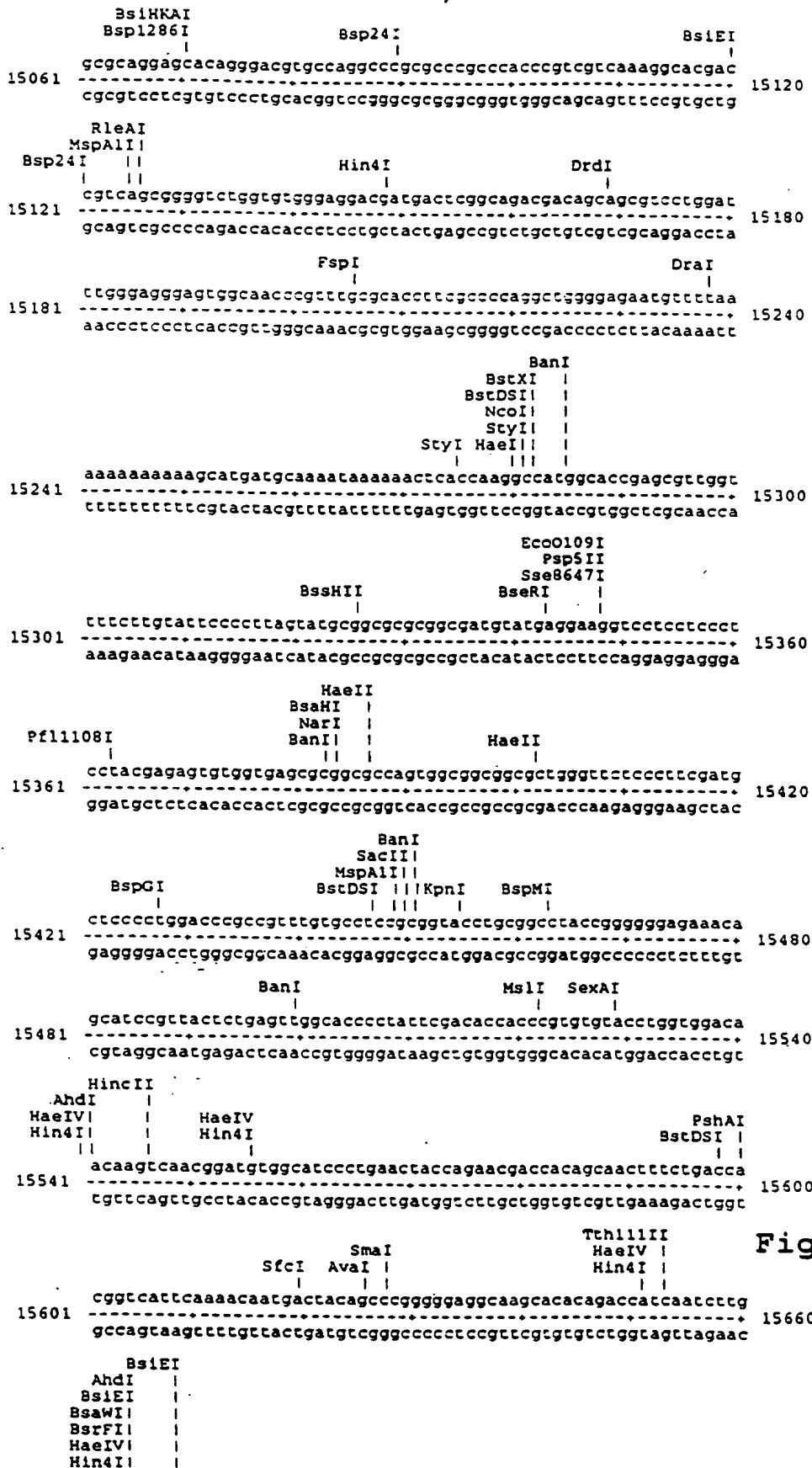


Figure 28W

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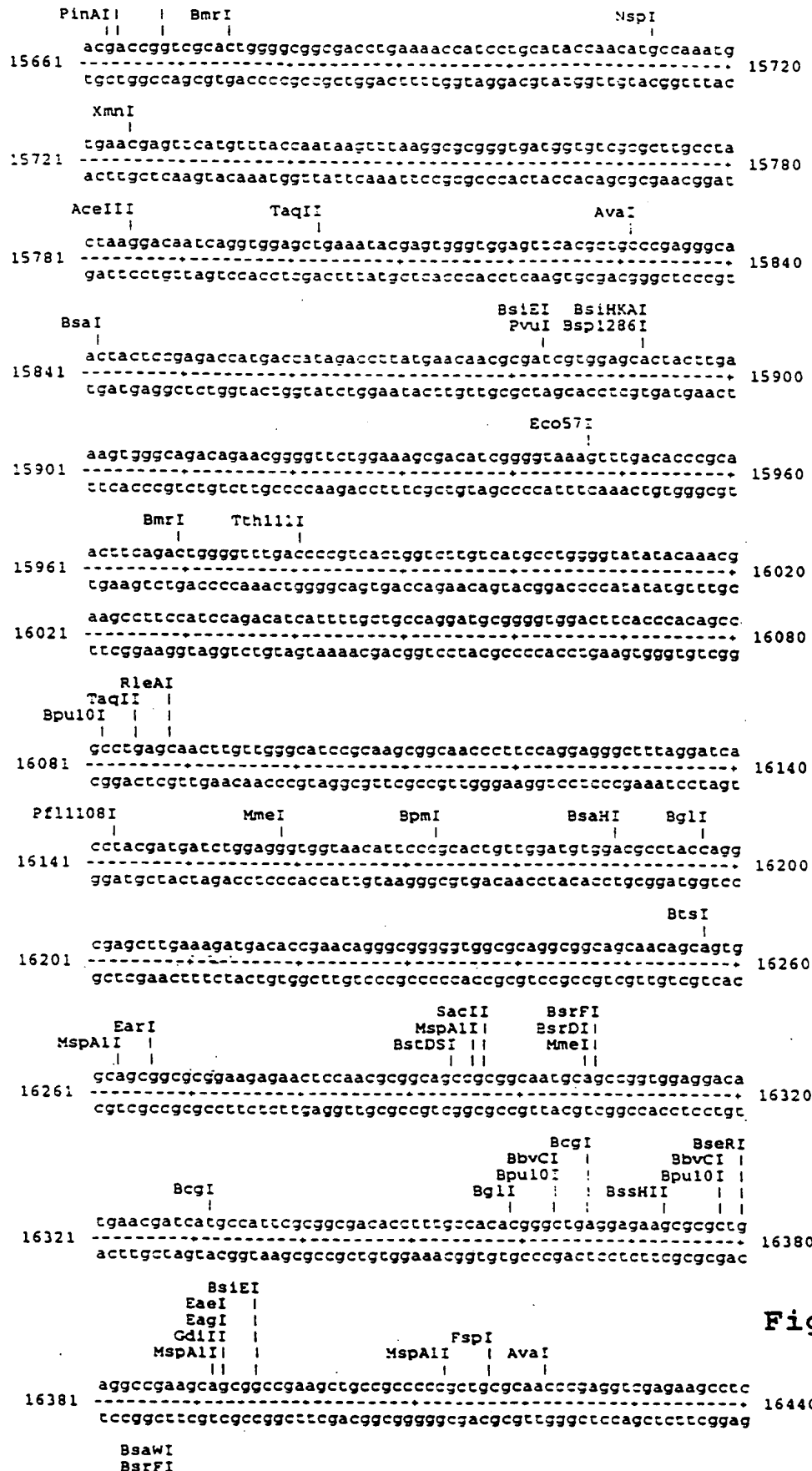


Figure 28X

SUBSTITUTE SHEET (RULE 26)

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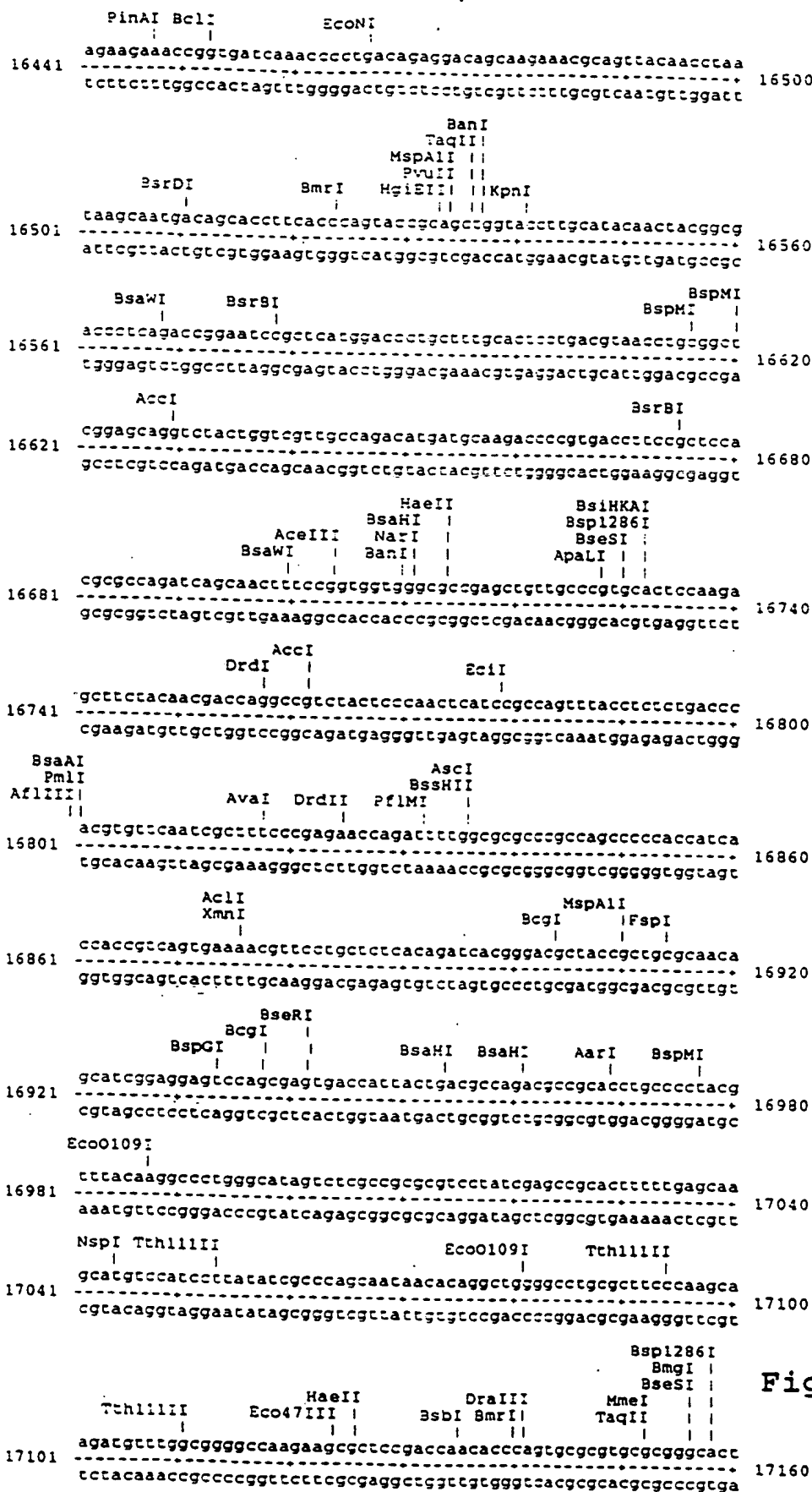


Figure 28Y

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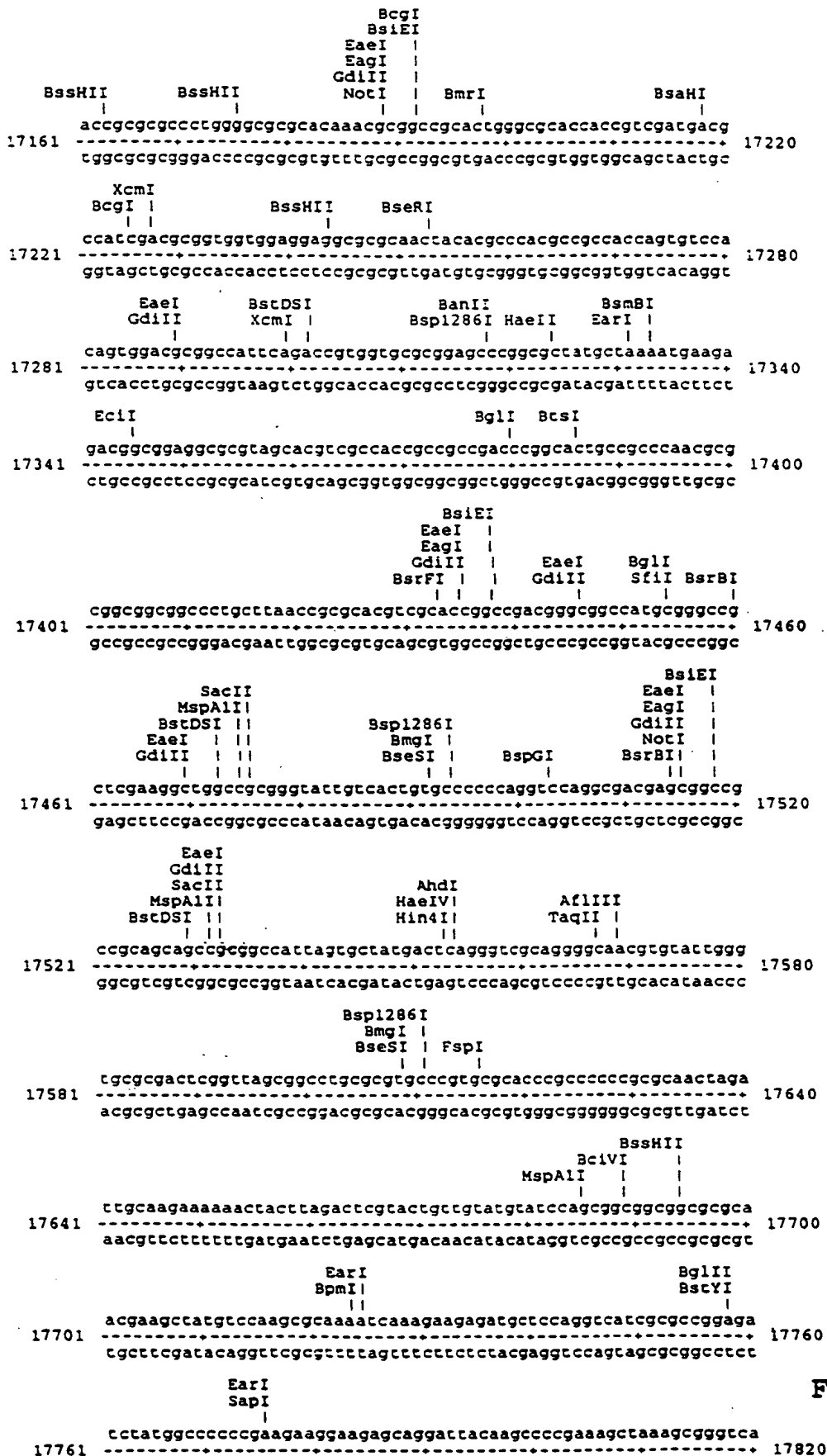


Figure 28Z

Figure 28AA

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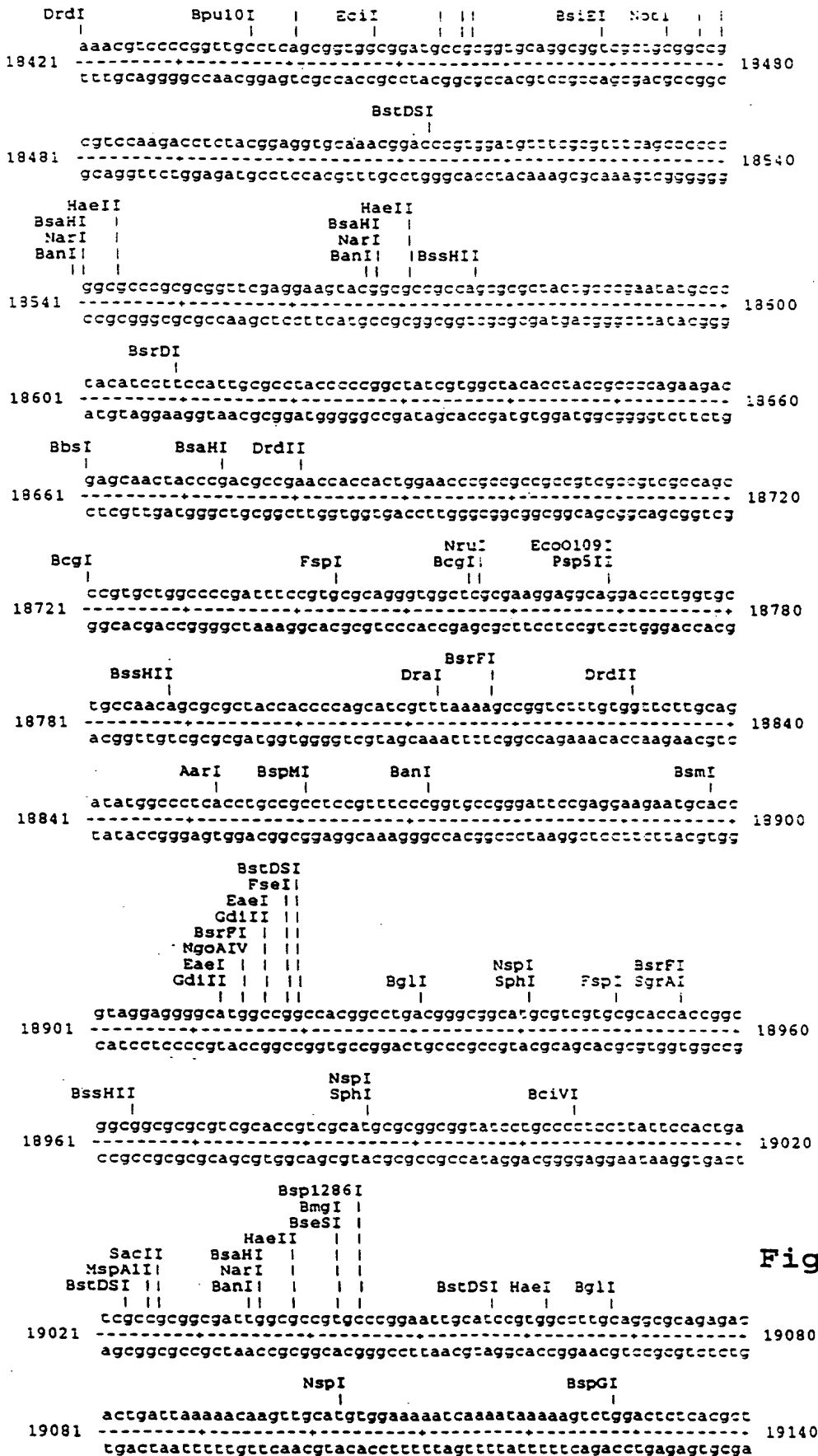


Figure 28BB

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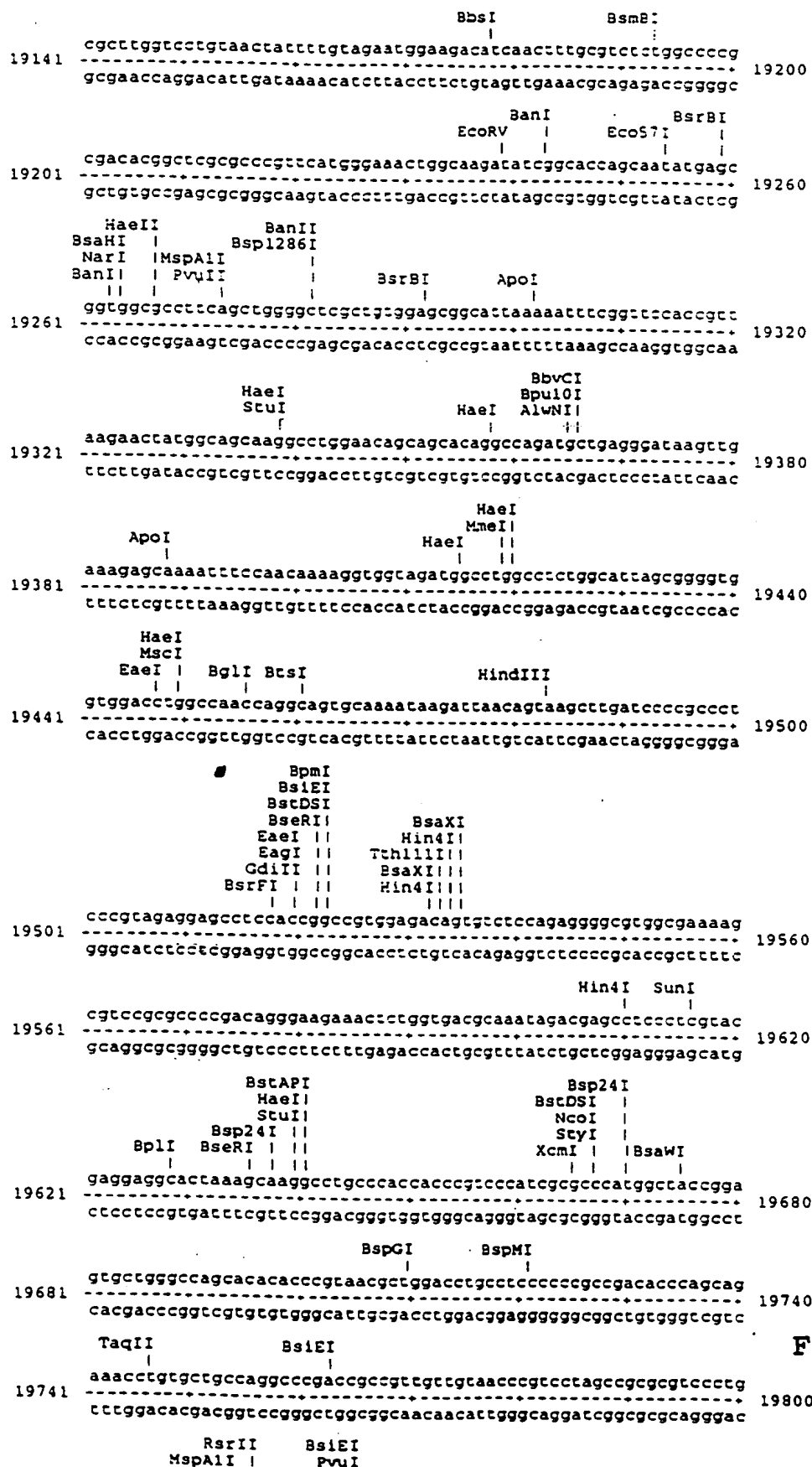


Figure 28CC

[illegible]

Figure 28DD

64/85

20461 acaacgaagacgaagtagacgagcaagctgagcagcaaaaaactcacgtatccyygcagg 20520
tgctgcttctgcttcattctgctcgctcgactcgctgcttttttgagtgcataaacccgccc

HaeII SspI
20521 cgcccttattctgggtataaatattacaaaggagggtattcaaatagggtgtcgaagggtcaaa 20580
gcggaataagaccatatttataatgtttccctcccataagtttatccacagcttccagttt

Tth1111I ECONI
20581 caccataaatatgccgataaaaacatttcaacctgaacctcaaataggagaatctcagtggt 20640
gtggatttatacggctattttgtaaagtggacttggagttatccctcttagagtcacca

VspI MspAI
PvuII
20641 acgaaactgaaattaatcatgcagctgggagagtccttaaaaagactaccccaatgaaac 20700
tgctttgactttaattagtagctcgacctcttcaggaattttctgatggggttactttg

NdeI RleAI BsmI
20701 catgttacgggttcattatgcaaaacccacaaatgaaatggagggtcaaggcattcttgtaa 20760
gtacaatgccaggtatagcttttgggtggttacttttacctcccggtccgtgaagaacatt

20761 agcaacaaaaatggaaagctagaaagtcaagtggaaatgcaatttttctcaactactgagg 20820
tcgttcttttaccttctgatcttctagttcacctttacgttaaaaagagttgatgacctc

BsiEI BsrDI BsrGI
TatI
20821 cgaccgcaggcaatgggtgataacttgactcctaaagtgggtattgtacagtgaagatgtag 20880
gctggcgctccgttaccactattgaactgaggatttaccataaacatgtcacttctacatc

NspI BssSI
20881 atatagaaacccagacactcatatttcttacctgcccactattaaggaaggtaactcac 20940
tatactctttggggtctgtgagtcataaagaatgtacgggtgataattccttccattgagtcg

HaeI
StuI
BglI BsrDI
20941 gagaactaatgggccaacaatctatgcccaacaggcctaattacattgcttttagggaca 21000
ctcttgattacccgggtgtgtgagacacgggttctccgattaatgtaacgaaaaatccctgt

TaqII
21001 attttattgggtctaattgtattacaacagcacgggtaatatgggtgtctctggcgggccaag 21060
taaaataaccagattacataatgttctgtctgcccattataccacaagaccgcccgttc

BsmI Tth1111I
21061 catcgcagttgaatgctgtttagatttgcaagacagaaacacagagctttcataccagc 21120
gtagcgtcaacttacgacaacatctaaacgttctgtcttctgtctctgaaagtatggtcg

DrdII
SexAI HincII
21121 ttttgcttgattccattgggtgatagaaccagggtactttctatgtggaatcaggctgttg 21180
aaaaagaaactaaggtaaccactatcttgggtccatgaaaagatacaccttagtccgacaac

21181 acagctatgatccagatgttagaattattgaaaaatcatggaactgaagatgaacttccaa 21240
cgtcgatacttaggtctacaatcttaataacttttagtaccttgacttctacttgaagggt

Eco57I BmrI VspI StyI
21241 attactgctttccactgggagggtgtgattaatcacagagactcttaccaaggtaaaacctta 21300
caatgacgaaaagggtgacctccacacctaattatgtctctgagaatgggtccattcttggat

EcoNI SfiI ApoI MmeI
21301 aaacagggtcaggaaaaatggatgggaaaaagatgctacagaattttcagataaaaaatgaaa 21360
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Figure 28EE

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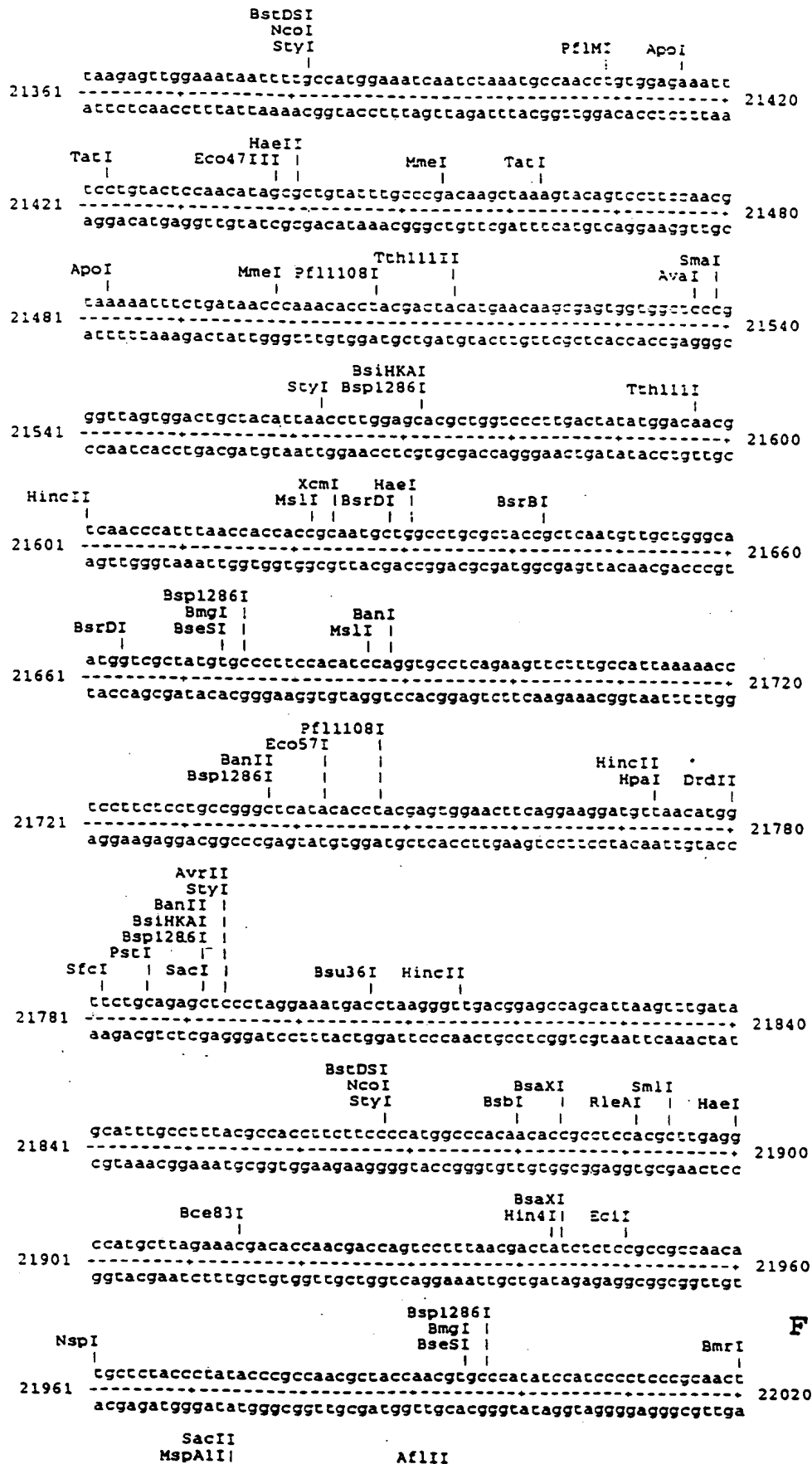


Figure 28FF

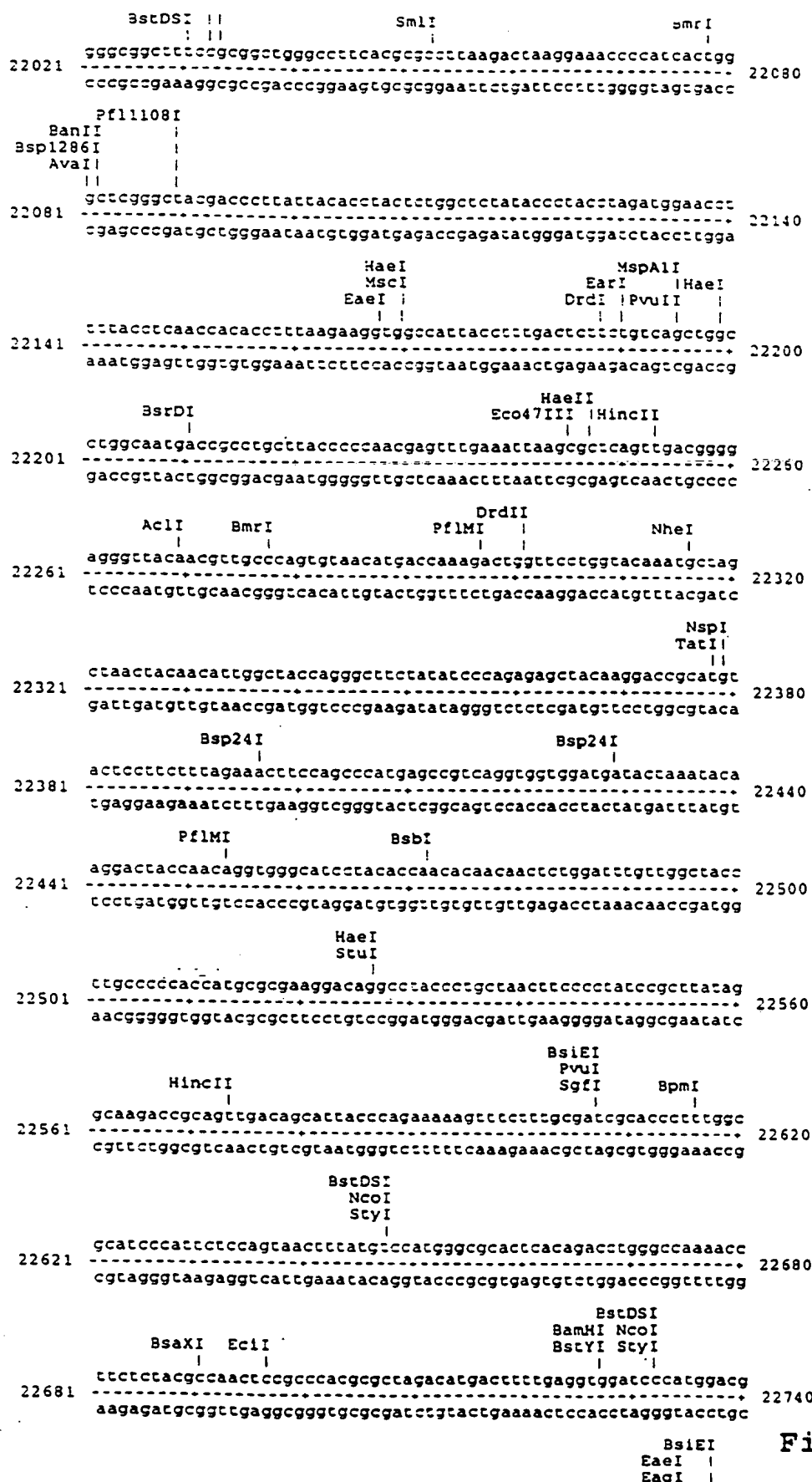


Figure 28GG

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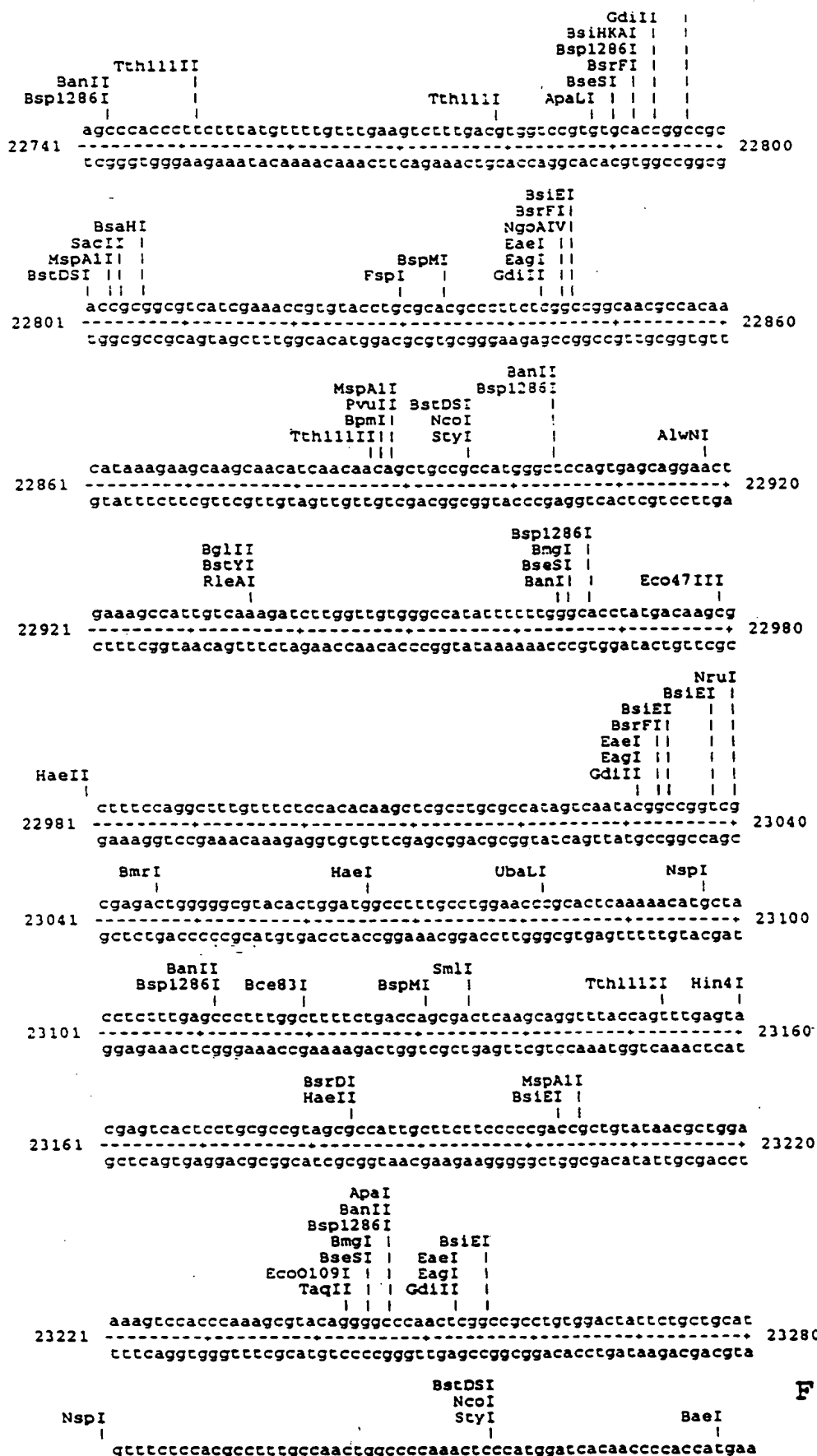


Figure 28HH

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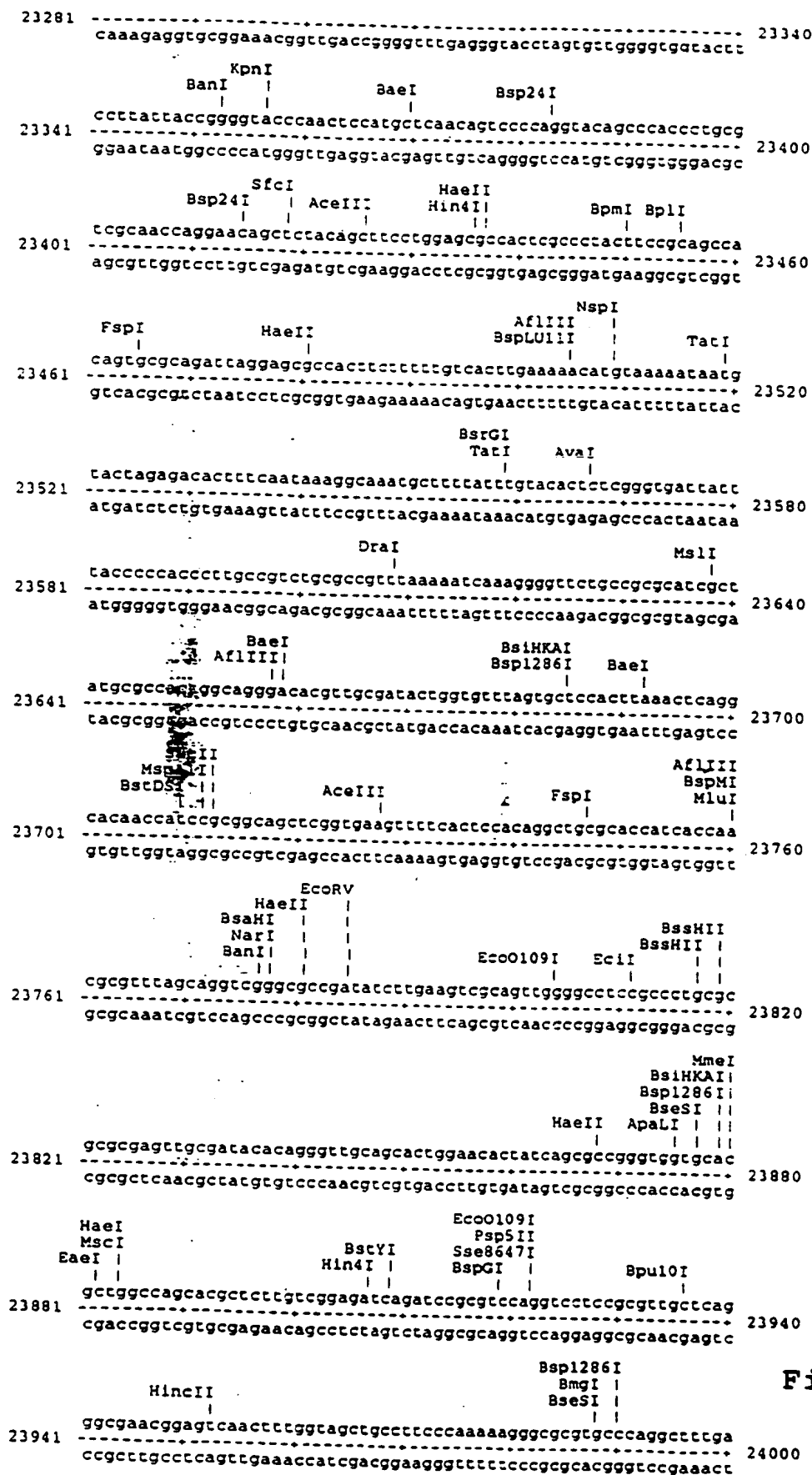
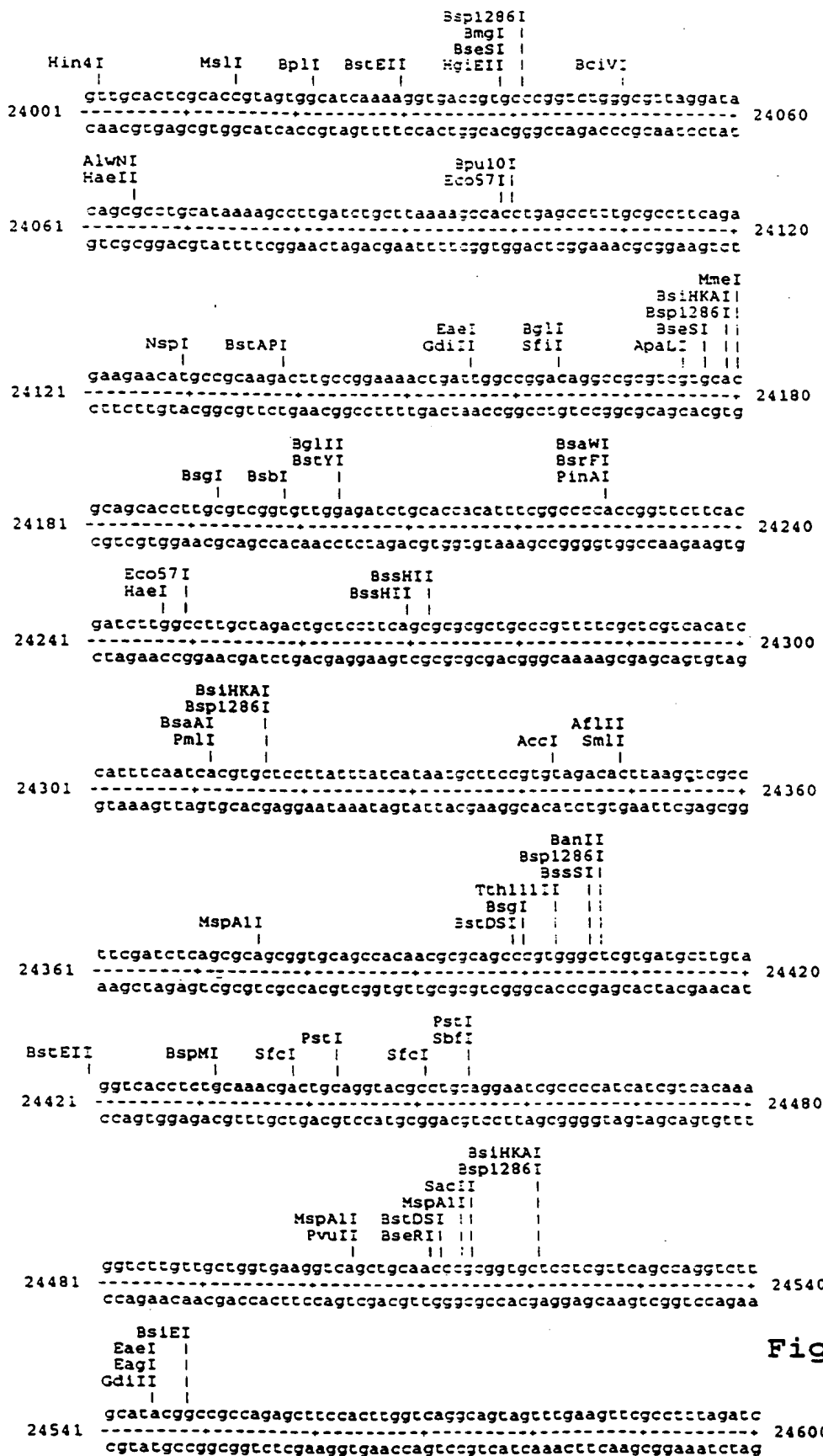


Figure 28II

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BssHII

Figure 28JJ

[illegible]

Figure 28KK

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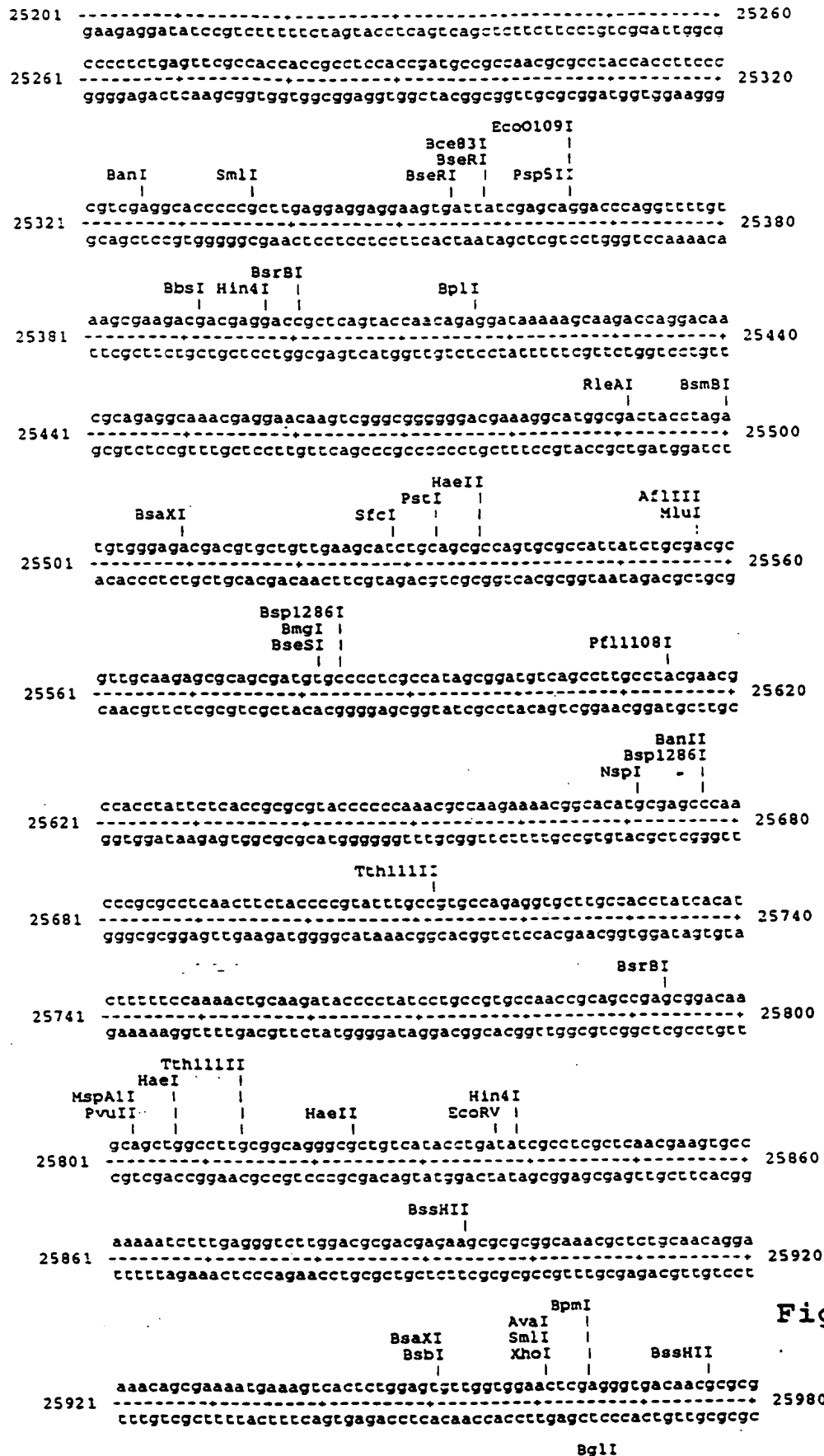


Figure 28LL

BstEII | TaqII
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 RcaI
 MslII
 AceII
 BsiHKA
 Bsp1286I
 HgiEII
 StyI
 RcaI
 FspI
 AlwNI
 26041 ----- 26100
 accccccaaggtcatgagcacagtcatgagtgagctgatcgtgcgcgctgctgcgagccccc
 tgggggggtccagtagctcgtgctcagtagctcactcgactagcacgcggcacgcgtcgggga
 Tth11111
 ApoI
 BpmI
 EcoO109I
 BseRI
 26101 ----- 26160
 ggagagggtgcaaatcttgcagaacaacagaggaggccctaccgcagctggcgacga
 cctctccctacgctttaaagcgtcttctgttctctcccgatggcgctcaaccgctgct
 BssHII
 NheI
 BseRI
 26161 ----- 26220
 gcagctagggcgctggcctcaaacgcgcgagcctgcgacttggaggagcgacgcaaacct
 cgtcgatcgcgcgacccaaggttgcgcgctcggacggctgaacctctctgctgcgtttga
 BsiHKA
 Bsp1286I
 BsaXI
 MspAI
 NspI
 SphI
 Bce83I
 EaeI
 GdiII
 BtsI
 BstDSI
 SmlI
 26221 ----- 26280
 aatgatggccgcagctgctcgtaccgctggagcttgagtgcatgcagcggtctcttctgctga
 ttactaccggcgctcacgagcaatggcacctcgaactcacgtacgtcgccaagaacacgact
 BsaAI
 SnaBI
 SunI
 BsrDI
 26281 ----- 26340
 cccggagatgcagcgcaagcttagaggaaacattgcactacacctttcgacagggtctacgt
 gggcctctacgtcgcgttcgactctcttttctaactgtagtggaagctgtcccgatgca
 BanII
 BsiHKA
 Bsp1286I
 MmeI
 BsaXI
 StyI
 ApoI
 HaeI
 BglII
 StuI
 BstYI
 SacI
 SexAI
 BsaI
 26341 ----- 26400
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 tgcggtccggacgtctctagaggtgcacctcgagacgttggaacagaggatggaacctta
 Bce83I
 StyI
 BstAPI
 SmlI
 AscI
 BssHII
 26401 ----- 26460
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 aaacgtgcttttggcggaaccgctttgcacgaagtaagggtcgagttcccgctccgccc
 BstDSI
 NcoI
 StyI
 EaeI
 GdiII
 26461 ----- 26520
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 ggcgctgatgcaggcgctgacgcaaatgaataaagatcagatgtggacgtctgcccggta
 AlwNI
 BstAPI
 PstI
 BseRI
 SfiI
 AceII
 BtsI
 Tth11111
 26521 ----- 26580
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 cccgcaaacctgcgtcacgaacctctccacgttggagttcctcgacgtcttgcagattc
 EaeI
 GdiII
 BstDSI
 HaeII
 Eco47III
 BsaXI
 EcoO109I
 Psp5II
 Sse8647I
 EcII
 gcaaaacttgaaggacctatggacggccttcaaacgagcgctccgtggccgcgcacctggc

Figure 28MM

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26581 ----- 26640
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 EcoNI AlwNI
 26641 ----- 26700
 ggacatcatctttcccgaaacgcctgcttaaaacccctgcaacagggtcttgccagacttcac
 cctgtagtaaaaggggcttgccgacgaattttgggacgttgcctccagacggtctgaagtg
 Bpu10I
 NspI HaeII
 Eco47III
 26701 ----- 26760
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 BsiHKA I
 Bsp1286 I
 BseSI
 BspMI
 AarI ApaLI
 Bsp1286 I
 BmgI
 BseSI
 EciI
 BsmI
 26761 ----- 26820
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 NheI
 PstI
 BtsI
 SfcI
 26821 ----- 26880
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 BsrBI
 BbsI
 AccI
 BstAPI
 BpmI
 26881 ----- 26940
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 gtattacctctctgcactcgccactgccagatgacctcacagtgcacgacgacgttggatac
 BsrBI
 MspAII
 PvuII
 BanI
 AceIII
 26941 ----- 27000
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 SacII
 MspAII
 BstDSI
 AhdI
 HaeIV
 Hin4I
 EcoO109I
 Psp5II
 SanDI
 PstI
 KpnI
 SfcI
 27001 ----- 27060
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 AatII
 BsaHI
 ApoI
 Bsu36I
 27061 ----- 27120
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 gtgaggecccgacacctgcagccgaatggaagcgtttaaactggactcctgatgggtgcg
 Pfl1108I
 BssSI
 UbaLI
 BbsI
 27121 ----- 27180
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 ggtgctctaatccaagatgcttctgggttagggcgggcggtttacgcttcgaatggcgga
 MunI
 HaeI
 MscI
 EaeI
 BstXI
 27181 ----- 27240
 cgtcattaccaggggccacattcttggcccaattgcaagccatcaacaaagcccgccaaga
 gcagtaatgggtcccggtgtaagaaccgggttaacggttcggtagttgttcggggcggttct
 Bmri
 AhdII
 HaeIV
 Hin4I
 BanII
 BsiHKA I
 Bsp1286 I
 SacI
 Pfl1108I
 gtttctgctacgaaagggacggggggtttacttggacccccagtcggcgaggagctcaa

Figure 28NN

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27241 ----- 27300
caaagacgatgctttccctgcccccaaatgaacctgggggtcaggccgctcctcgagtt
ApaI
BamII
Bsp1286I
BmgI
BseSI
EcoO109I
SacII
MspAII
BstDSI
BseRI
cccaatccccccgcccgcgcagccctatcagcagcagccgcgggccccttgcttcccagga
27301 ----- 27360
gggttaggggggaggcggtcggtatagtcgtcggtcgcccggaacgaagggtcct
MspAII
PvuII
PstII
TaqII
SfcI
BstDSI
TaqII
BseRI
BamRI
tgccacccaaaaagaagctgcagctgcccgcgcacccacggacgaggaggaataactggg
27361 ----- 27420
accgtgggtttttcttcgacgtcgacggcggtgggtgcttcttcttatgacct
BseRI
BseRI
BseRI
BbsI
BamRI
acagtcaggcagaggaggttttggacgaggaggaggacacgacggaagactgggaga
27421 ----- 27480
tgtcagtcctgtctctccaaaacctgctcctcctcctcctgtactaccttctgacctct
HindIII
EcoNI
EarI
TaqII
BcgI
gcctagacgaggaagcttccgaggtcgaagaggtgtcagacgaaacacctcaccctcgg
27481 ----- 27540
cggatctgtctcttcgaaggctccagcttctccacagtctgcttctggcagtgaggagcc
HaeII
BsaHI
NarI
BamII
BsrFI
NgoAIV
SgrAI
BcgI
BsaWI
BsrFI
PnaI
BseRI
tcgcattccccctcgccggcgccccagaaatcggaaccgggttcagcatgggtacaacct
27541 ----- 27600
agcgttaaggggagcgccgggggtcttttagccgttgccaagggtcgtaccgatgttga
BsrFI
NgoAIV
HaeII
BsaHI
Bsu36I
BsrBI
NarI
BsaXI
BamII
BtsI
ccgctcctcaggcgccgcggcactgcccgttcgcccagcccaaccgtagatgggacacca
27601 ----- 27660
ggcgaggagtcgcgggcgccgtgacgggcaagcgggtgggttggtatctacctgtgtg
BsrFI
HglEIII
DrdII
Tth111II
ctggaaccagggcggttaagtcgaagcagccgcccgttagcccaagagcaacaacagc
27661 ----- 27720
gaccttgggtcccgcccatcaggttcgtcggtcgccggcggaatcggttctcgttctgtcg
Tth111II
Tth111II
Bsp1286I
BmgI
BseSI
RleAI
HaeII
SlyI
BsrBI
gccaaggtaccgctcatggcgcgccgacacaagaacgccatagttgcttgcctgcaagacc
27721 ----- 27780
cggttccgatggcgagtagccgccccgtgttcttgcgggtatcaacgaacgaacgttctga
HaeI
DraII
gtgggggcaacatctcttcgcccggcgttcttctctaccatcacggcggtggccttcc
27781 ----- 27840
caccctcggttagaggaagcgggcgccggaagaagagatggttagtgcgcacccggaagg

Figure 2800

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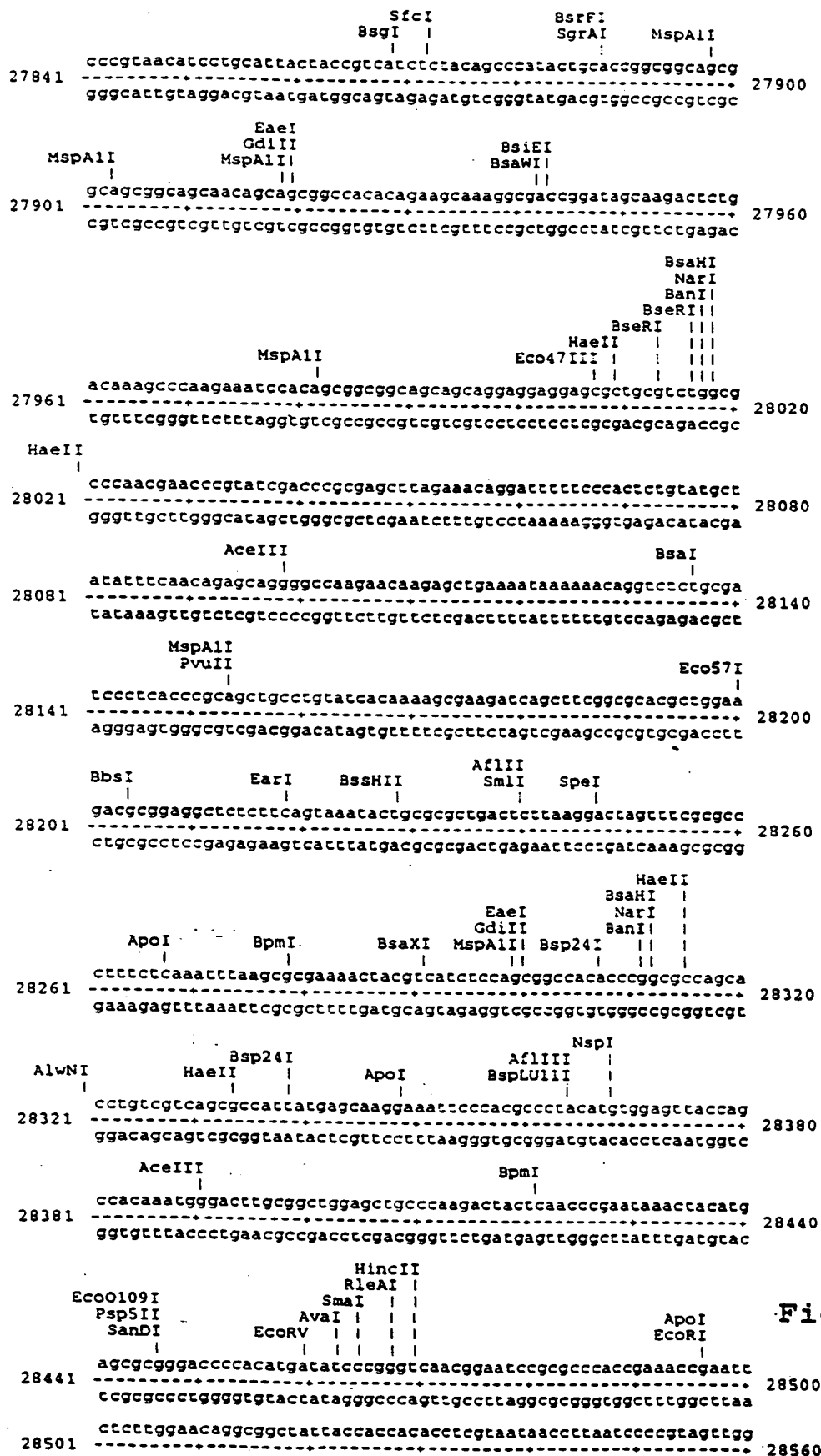


Figure 28PP

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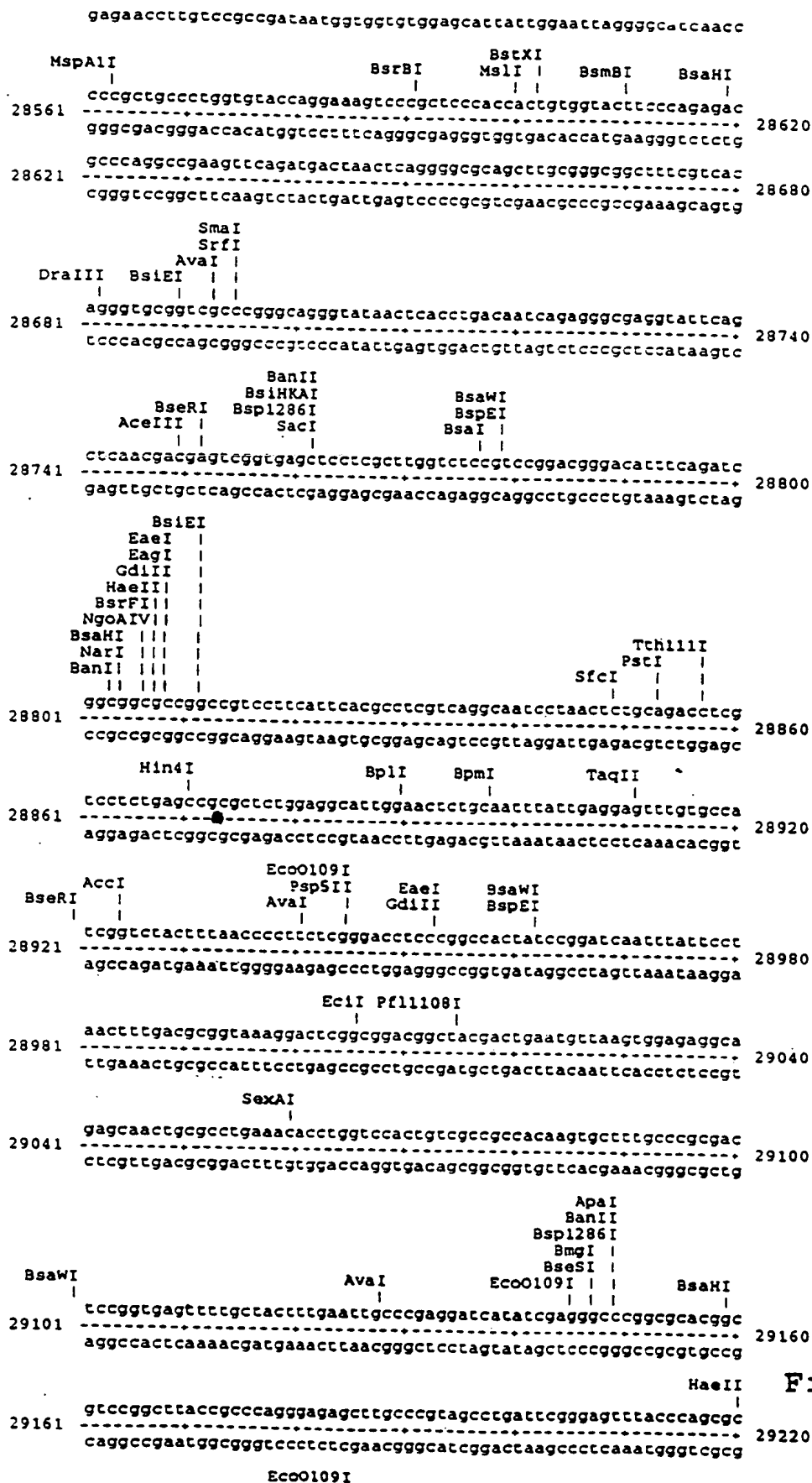


Figure 28QQ

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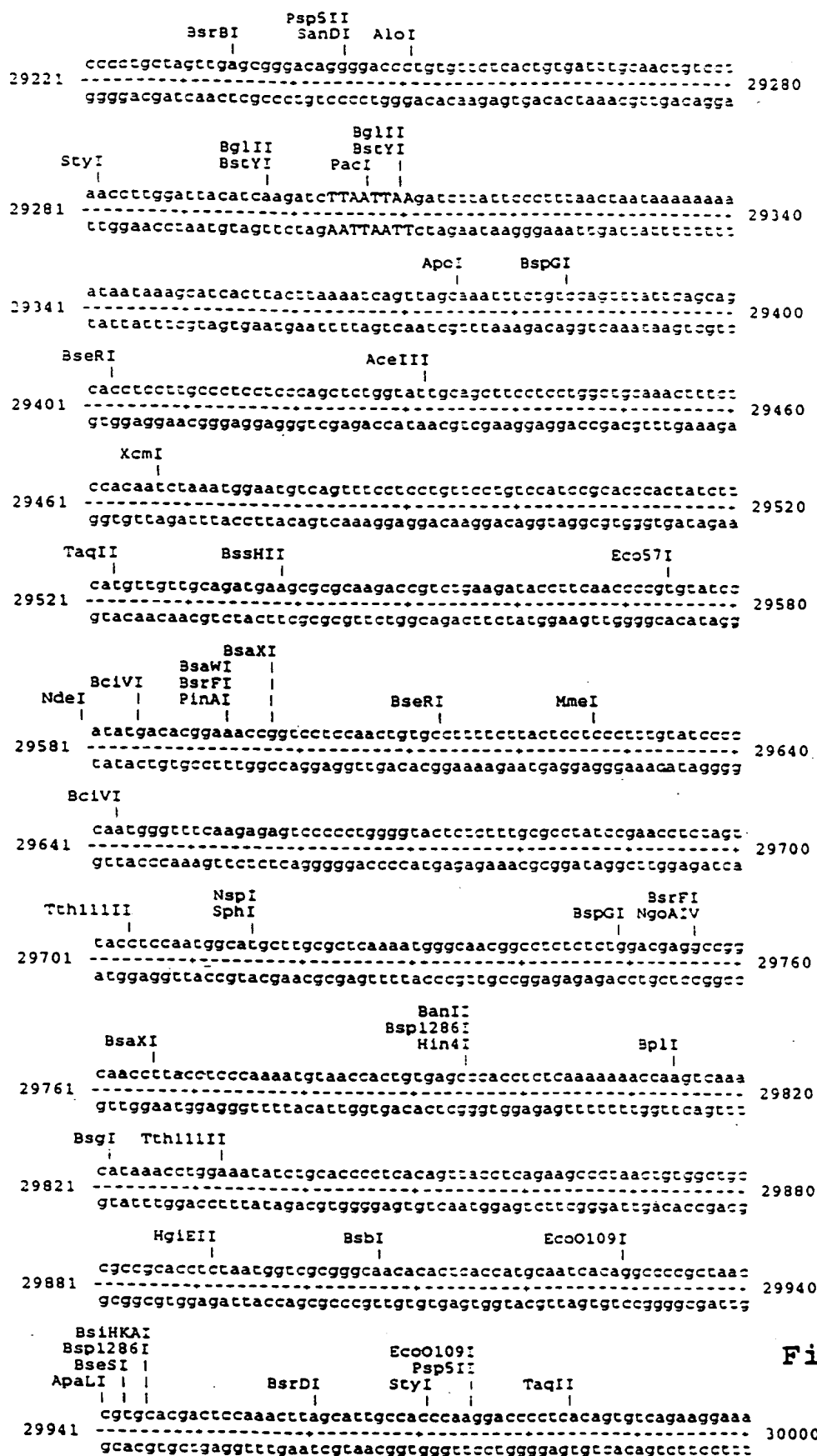


Figure 28RR

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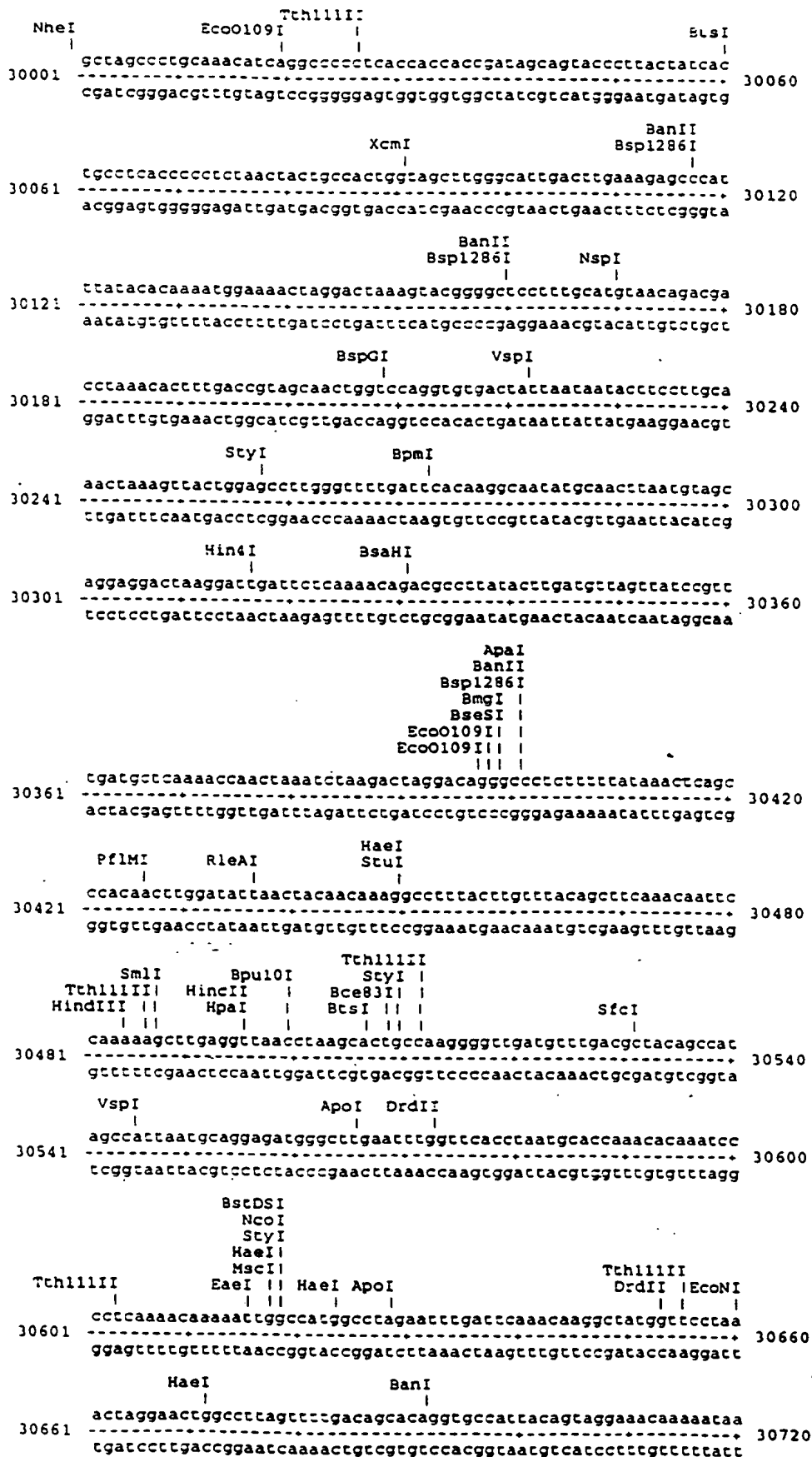


Figure 28SS

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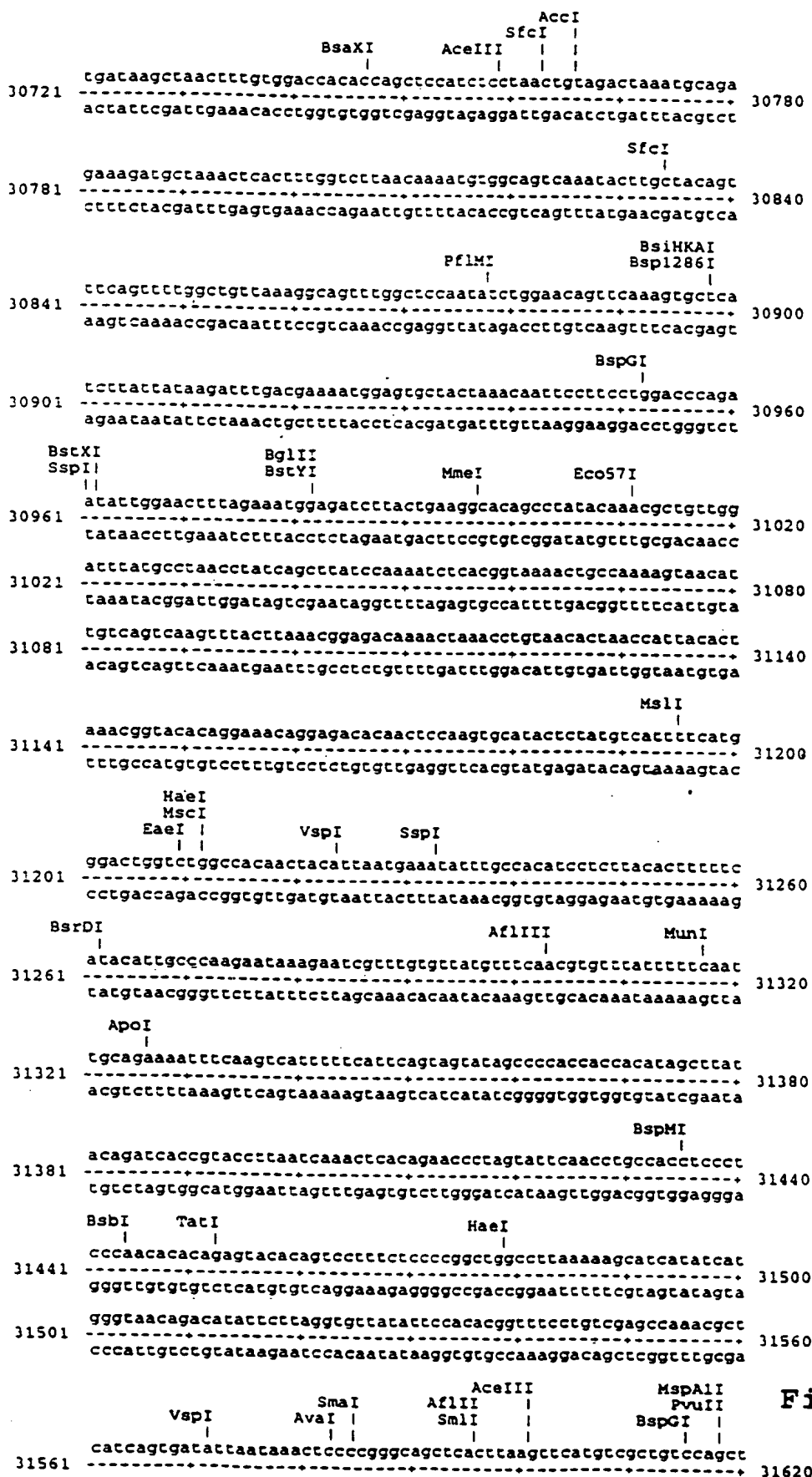


Figure 28TT

80/85

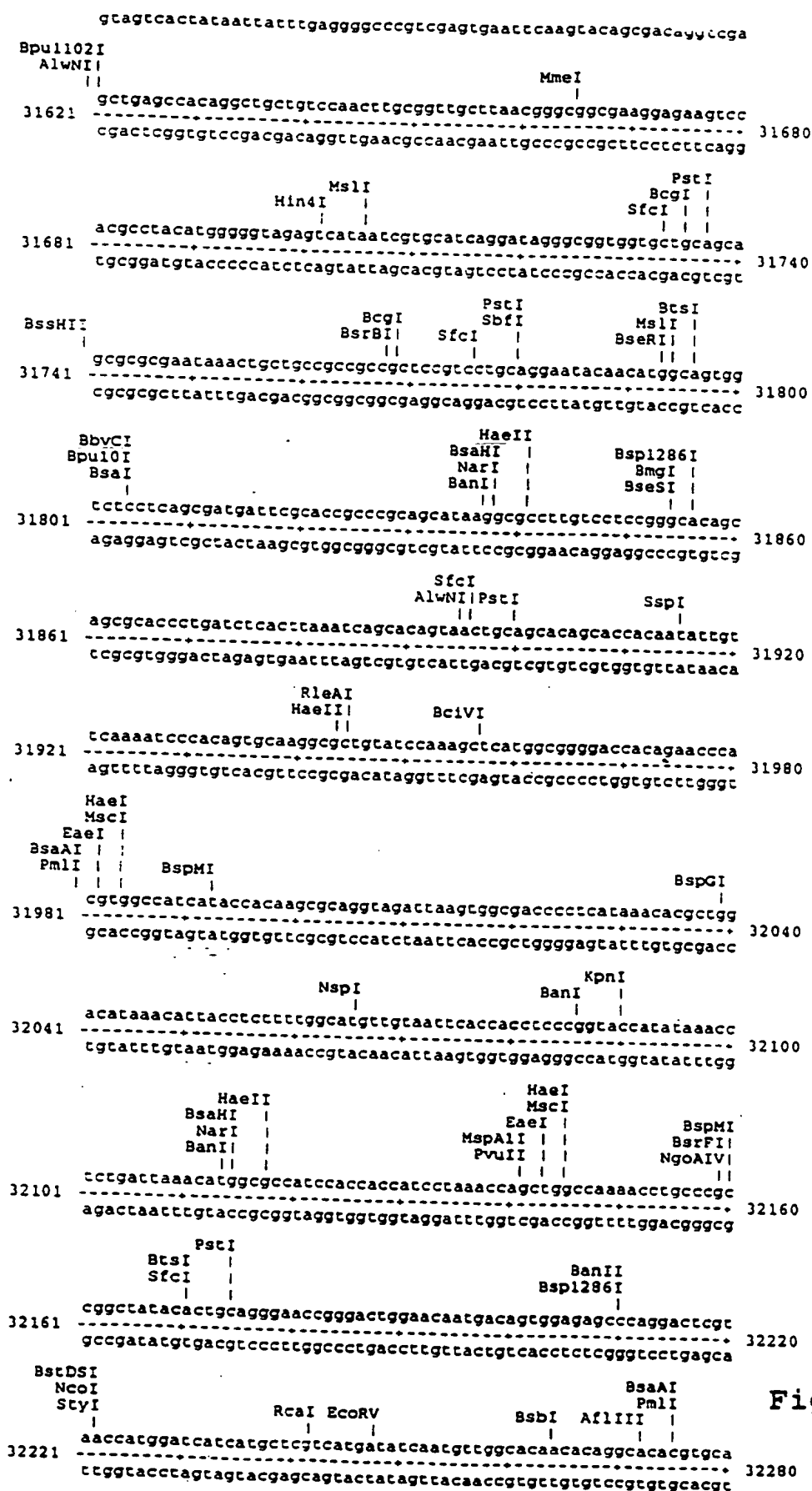


Figure 28UU

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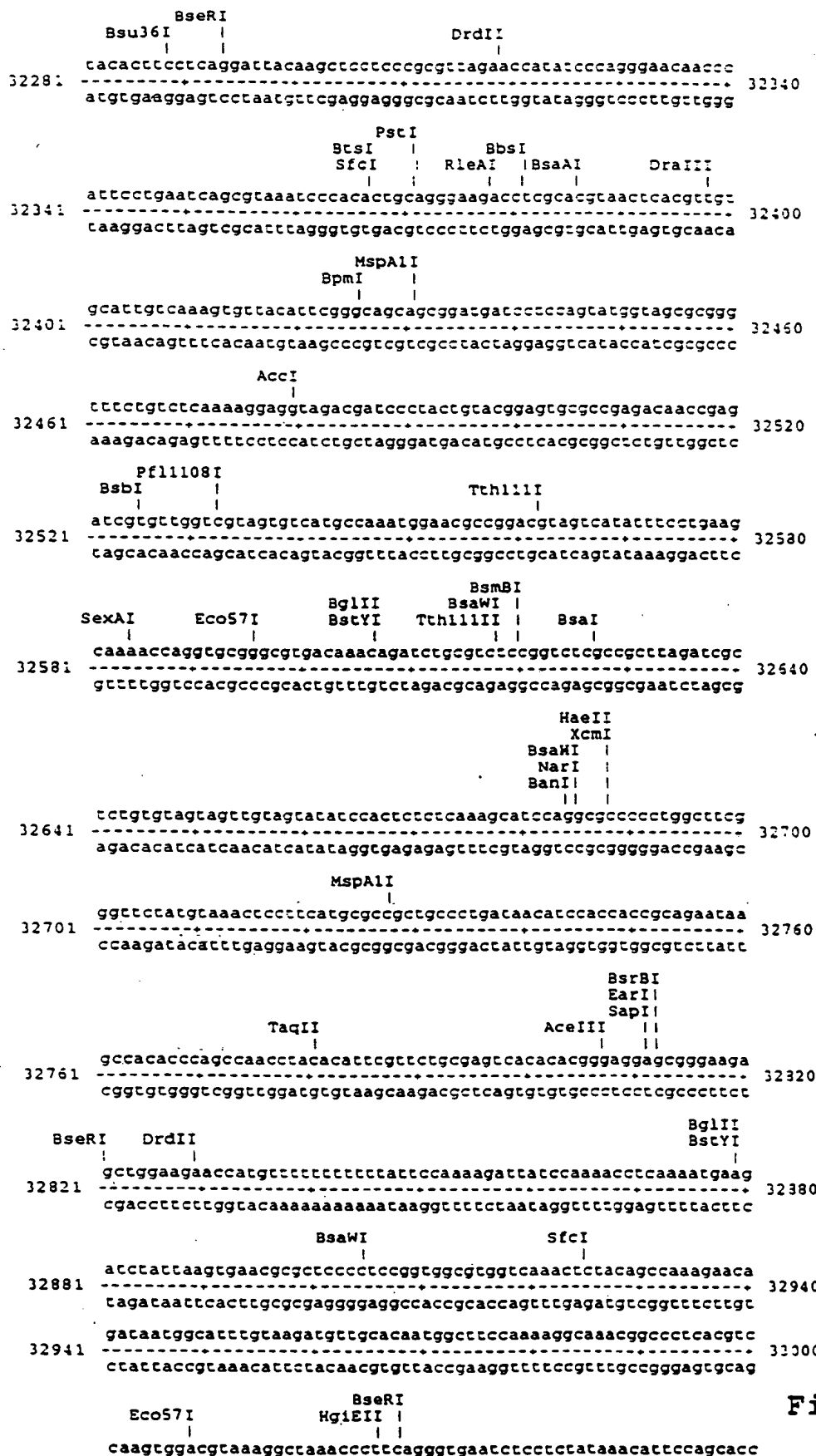


Figure 28VV

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33001 ----- 33060
gttcacctgcatttcggtttgggaagtcctccttagaggagatattgttaaggctcgtgg

33061 tccaaccatgccccaaataattctcatctcgccaccttctcaatatattcttaagcaaac
----- 33120
aagttgggtacgggtttattaagagtagagcggcgaagagcttatatagagattcgttttag

33121 ----- 33180
ccgaatattaagtccggccatttgtaaaaaatctgctccagagcgcctccaccttcagcct

ggctttataattcaggccggtaacatttttagacgaggtctcgcgggaggtggaagtcgga

33181 ----- 33240
caagcagcgaatcatgattgcaaaaattcagggtctctcacagacctgtataagattcaaa

gttcgctcgttagtactaacgtttttaagtcgaaggagtgctggacatattcttaagttt

33241 ----- 33300
agcgggaacatttaacaaaaataccgcgatcccgtaggtcccttcgcagggccagctgaaca

tcgccttgtaattgtttttatggcgctagggcatccagggaagcgtcccggtcgacttct

33301 ----- 33360
taatcgtgcaggtctgcacggaccagcgcggccacttccccgcaggaaccttgacaaaa

attagcacgtccagacgtgcctggcgcgcggtgaagggcggtccttggaactgtttt

33361 ----- 33420
gaacccacactgattatgacacgcatactcggagctatgctaaccagcgtagccccgatg

cttgggtgtgactaatactgtgcgtatgagcctcgatcagactggctcgcatcggggctac

33421 ----- 33480
taagctttgttcgatggcgccgatataaaatgcaagggtgctgctcaaaaaatcaggcaa

attcgaaacaacgtaccgcgcgtatattttacgttccacgacgagtttttagtccgtt

33481 ----- 33540
agccttcgcgcaaaaaagaaagcacatcgttagtcatgctcatgcagataaaggcaggtaag

tcggagcgcgtttttttttctgtgtagcatcagtagcaggtacgtctatttccgtccattc

33541 ----- 33600
ctccggaaccaccacagaaaaagacaccattttttcttcaaacatgtctcgggtttcttg

gaggcctcgttggtgtctttttctgtggttaaaaagagagtttctacagacgccccaaagac

33601 ----- 33660
cataaacacaaaaataaaatacaaaaaaacatttaaacattagaagcctgtcttacaaca

gtatttctgtttttattttattgttttttgtaaatcttcggacagaatgttgt

33661 ----- 33720
ggaaaaacaacccttataagcataagacggactacggccatgccggcgtgaccgtaaaaa

cctttttctgggaataattcgtattctgcctgatgccgggtacggccgactggcattttt

33721 ----- 33780
aactgggtcacctgattaaaaagcaccaccgacagctcctcgggtcatgtccggagtcata

ttgaccagtgccactaatctttctgtggtggctgtcaggagccagtagcaggcctcagtag

33781 ----- 33840
atgtaagactcggtaaacacatcagggttgattcatcggtcagtgctaaaaagcggaccgaa

cacattctgagccattctgttagtccaactaaagtagccagtcacgatttttctgtggctt

Figure 28WW

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TaqII
 SmaI
 AvaI
 33841 atagcccgggggaatacatacccgaggcgtagagacaacattacagcccccattaggagg 33900
 tatcgggcccccttatgtatggggtccgcatctctgttgaatgtcgggggtatcctcc
 VspI
 AvrII
 StyI
 33901 tataacaaaattaatagagagagaaaaacacataaacacctgaaaaacctctccttagg 33960
 atattgttttaattatcctctctctctctgtgtattctgtggaacttttgggaggacggatcc
 BpmI BsrBI HaeII Eco47III MspAII
 33961 caaaatagcaccctcccgctccagaacaacatacagcgcttcacagcggcagcctaacag 34020
 gttttatcgtgggagggcgaggtcttctgtgtatgtcgcgaagtgtcgcctcggattgtc
 Bani
 34021 tcagccttaccagtaaaaaagaaaacctattaaaaaacaccactcgacacggcaccagc 34080
 agtcggaatgggtcattttttctcttggataattttttgtggtgagctgtgcctggtcgtc
 AceIII BspI
 34081 tcaatcagtcacagtcgtaaaaaagggccaaagtcagagcgagttatatataggactaaaaa 34140
 agttagtcagtcacacatttttcccggttcacgtcttcgtcatatatatcctgattctt
 TaqII
 34141 atgacgtaacgggttaaagtcacaaaaaacaccagaaaaaccgcacgcgaacctacgccc 34200
 tactgcattgcgaatttcagggtgtttttgtgggtcttttggcgtgcgcttggatgcggg
 RleAI
 34201 agaaacgaaagccaaaaaacccacaacttctcctcaaatcgtcacttccgttttcccacgtt 34260
 tctttgcttccggttttttgggtgttgaaggagtttagcagtcgaaggcaaaaggggtgcaa
 BsaAI SnaBI BsbI EciI
 34261 acgtaacttcccattttaagaaaactacaattcccaacacatacaagttactccgccccta 34320
 tgcattcgaagggttaaaattcttttgatgttaagggttctgtcatgttcaatgaggcgggat
 34321 aaacctacgtcaccgcccccggttcccacgccccgcgcacgtcacaaaactccacccccctc 34380
 tttggatgcagtcggggcggggcaagggtcgggggcgcggtgcagtcgtttgaggtgggggag
 34381 attatcatattgggttcaatccaaaataagggtatattattgatgatg 34427
 taatagtcataaccgaaggttaggttttattccacataataactactac

Enzymes that do cut:

AarI	AacII	AccI	AceIII	AcII	AflIII	AflIII	AhdI
AloI	AlwNI	Apal	ApalI	ApoI	AscI	AvaI	AvrII
BaeI	BamHI	BanI	BanII	BbsI	BbvCI	Bce81I	BcgI
BclVI	BclI	BglI	BglII	BmgI	BmrI	BplI	BpmI
Bpu10I	Bpu102I	BsaI	BsaAI	BsaBI	BsaHI	BsaWI	BsaXI
BsbI	BseRI	BseSI	BsgI	BsIEI	BsHKAI	BsmI	BsmBI
Bsp24I	Bsp1286I	BspEI	BspGI	BspLUII	BspMI	BsrBI	BsrDI
BsrFI	BsrGI	BssHII	BssSI	BstAPI	BstDSI	BstEII	BstXI
BstYI	BstZ17I	Bsu36I	BtsI	Clal	DraI	DraIII	OrdI
DrdII	EaeI	EagI	EarI	EciI	Eco47III	Eco57I	EcoNI
EcoO109I	EcoRI	EcoRV	FseI	FspI	GdlII	HaeI	HaeII
HaeIV	HgiEII	Hin4I	HincII	HindIII	HpaI	KpnI	MluI
MmeI	MscI	MslI	MspAII	MunI	NarI	NcoI	NdeI
NgoAIV	NheI	NotI	NruI	NsiI	NspI	PacI	Pfl1108I
PflMI	PinAI	PmeI	PmlI	PshAI	Psp5II	PstI	PvuI
PvuII	RcaI	RleAI	RsrII	SacI	SacII	SalI	SanDI
SapI	SbfI	ScaI	SexAI	SfcI	SfiI	SgfI	SgrAI
SmaI	SmlI	SnaBI	SpeI	SphI	SrfI	Sse8647I	SspI
StuI	StyI	SunI	SwaI	TaqII	TatI	Tth111I	Tth111II
UbaLI	VspI	XbaI	XcmI	XhoI	XmnI		

Enzymes that do not cut:

NspV

Figure 28XX

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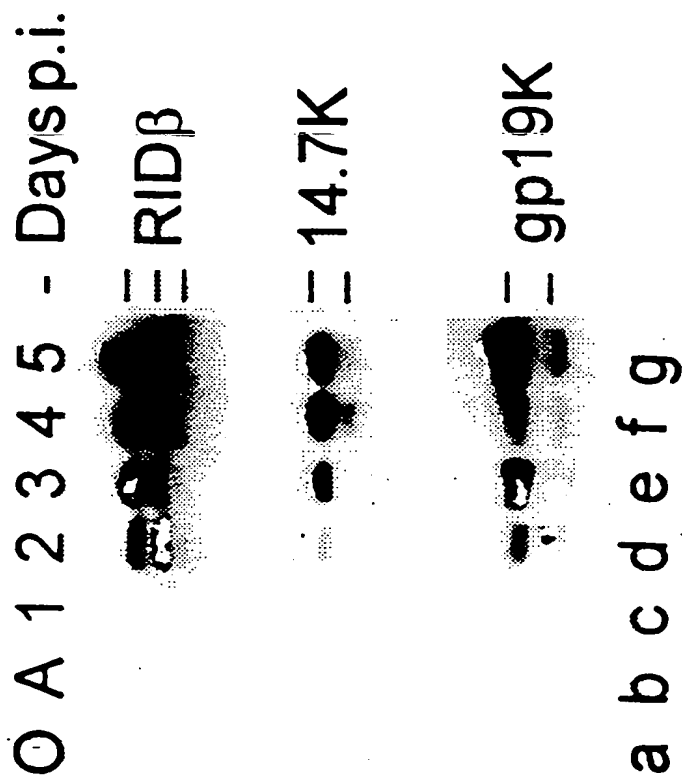


Figure 29

14.7K

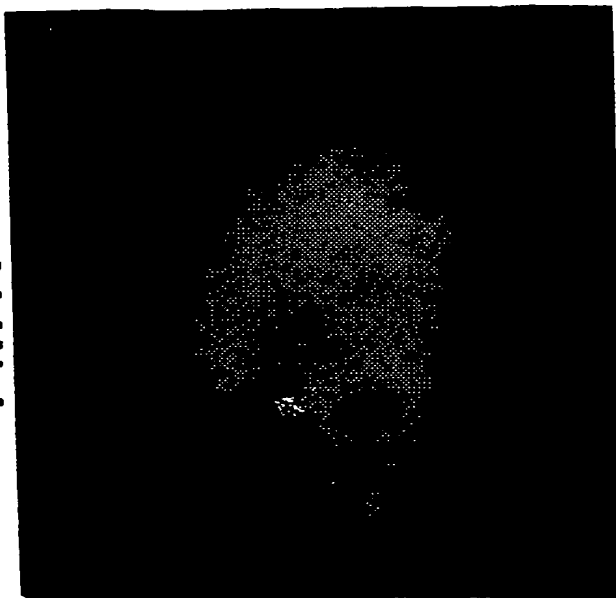


Figure 30C

RID β

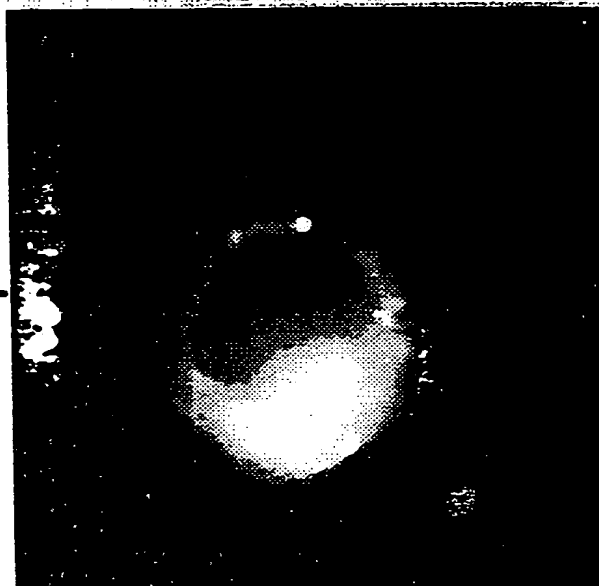


Figure 30B

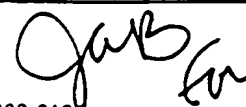
gp19K



Figure 30A

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/14239

A. CLASSIFICATION OF SUBJECT MATTER IPC(6) : C12N 7/01, 15/34, 15/87 US CL : 435/456, 372, 377 According to International Patent Classification (IPC) or to both national classification and IPC																				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/456, 372, 377 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) MEDLINE, BIOSIS, EMBASE, CANCERLIT, WPIDS, USPATFULL apoptosis, FAS, TNFR, DR3, TRAIL-R1, TRAIL-R2, leukocyte, transplant, adenovirus																				
C. DOCUMENTS CONSIDERED TO BE RELEVANT																				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																		
Y,P	US 5,674,734 A (LEDER et al) 07 October 1997, see entire document.	1-25																		
Y	GOODING, L.R. et al. The anti-viral activities of TNF: Adenovirus strikes back. J. Cell Biochem. 27 January-03 February 1990, Suppl. 0, Vol. 14, Part B, page 18, abstract CD34.	1-25																		
Y	GOODING, L.R. et al. Adenovirus genes that modulate the sensitivity of virus-infected cells to lysis by tumor necrosis factor. J. Cell. Biochem. 08 February 1992. Suppl. 0 Vol. 16, part B, page 269, abstract J005.	1-25																		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.																				
<table border="0"><tr><td>* Special categories of cited documents:</td><td>*T*</td><td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td></tr><tr><td>*A* document defining the general state of the art which is not considered to be of particular relevance</td><td>*X*</td><td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td></tr><tr><td>*E* earlier document published on or after the international filing date</td><td>*Y*</td><td>document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td></tr><tr><td>*L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td><td>*A*</td><td>document member of the same patent family</td></tr><tr><td>*O* document referring to an oral disclosure, use, exhibition or other means</td><td></td><td></td></tr><tr><td>*P* document published prior to the international filing date but later than the priority date claimed</td><td></td><td></td></tr></table>			* Special categories of cited documents:	*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	*E* earlier document published on or after the international filing date	*Y*	document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	*L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family	*O* document referring to an oral disclosure, use, exhibition or other means			*P* document published prior to the international filing date but later than the priority date claimed		
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E earlier document published on or after the international filing date	*Y*	document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																		
L document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family																		
O document referring to an oral disclosure, use, exhibition or other means																				
P document published prior to the international filing date but later than the priority date claimed																				
Date of the actual completion of the international search 20 AUGUST 1998		Date of mailing of the international search report 08 OCT 1998																		
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer MARY TUNG Telephone No. (703) 308-0198 																		

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/14239

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	ZHANG, H. et al. Amelioration of collagen-induced arthritis by CD95 (Apo-1/Fas)-ligand gene transfer. J. Clin. Invest. October 1997, Vol. 100, No. 8, pages 1951-1957, see entire document.	1-25